Identification and Characterization of Microbial Community in the Coelomic Fluid of Earthworm (*Aporrectodea molleri*)

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Received: April 26, 2021 / Revised: May 12, 2021 / Accepted: May 18, 2021

Earthworms play an important role in soil fertilization, interacting continually with microorganisms. This study aims to demonstrate the existence of beneficial microorganisms living in the earthworm's immune system, the coelomic fluid. To achieve this goal, a molecular identification technique was performed, using cytochrome c oxidase I (COI) barcoding to identify abundant endogenic earthworms inhabiting the temperate zone of Rabat, Morocco. Then, 16S rDNA and ITS sequencing techniques were adopted for bacteria and fungi, respectively. Biochemical analysis, showed the ability of bacteria to produce characteristic enzymes and utilize substrates. Qualitative screening of plant growth-promoting traits, including nitrogen fixation, phosphate and potassium solubilization, and indole acetic acid (IAA) production, was also performed. The result of mitochondrial COI barcoding allowed the identification of the earthworm species *Aporrectodea molleri*. Phenotypic and genotypic studies of the sixteen isolated bacteria and the two isolated fungi showed that they belong to the *Pseudomonas*, *Aeromonas*, *Bacillus*, *Buttiauxella*, *Enterobacter*, *Pantoea*, and *Raoultella*, and the *Penicillium* genera, respectively. Most of the isolated bacteria in the coelomic fluid showed the ability to produce β -glucosidase, β -glucosaminidase, Glutamyl- β -naphthylamidase, and aminopeptidase enzymes, utilizing substrates like aliphatic thiol, sorbitol, and fatty acid ester. Furthermore, three bacteria were able to fix nitrogen, solubilize phosphate and potassium, and produce IAA. This initial study demonstrated that despite the immune property of earthworms' coelomic fluid, it harbors beneficial microorganisms. Thus, the presence of resistant microorganisms in the earthworm's immune system highlights a possible selection process at the coelomic fluid level.

Keywords: *Aporrectodea molleri*, plant growth-promoting rhizobacteria (PGPR), coelomic fluid, earthworm.

Introduction

Earthworms belong to the *Annelidae*, family of *Lumbricidae*, class of *oligochaetes*, which evolved at the end of the Precambrian and includes more than 8000

species [1]. They occupy a unique position in the animal kingdom and have successfully invaded terrestrial habitats [2]. Moreover, earthworms play an important role in temperate terrestrial ecosystems; they fertilize soils by modifying their physicochemical and biological properties (soil texture, regulation of biogeochemical cycles, etc.) [1, 3–5]. In order to carry out these actions, earthworms must interact with other components of the soil ecosystem, such as microflora (bacteria, fungi, etc.)

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[6]. In several studies, it has been shown that soil-level transformations involve synergistic interactions between earthworms and microorganisms. Furthermore, the presence of earthworms in soil has a great influence on soil microbial community structure and activity [7]. This can be strongly related to earthworm's dispersal activity within the created burrows [8]. Nevertheless, because of their limited ability to disperse within the soil, a large proportion of soil microorganisms are inactive, at any time, waiting for suitable conditions to promote higher levels of activity [9]. The abundant population of earthworms in soil provide an ideal environment for enhanced activity levels or multiplication of some selected microorganisms in the gut. Therefore, the most microorganisms studied in earthworms were limited on microbial communities ingested with the soil and passed through the earthworm gut, considering the intestine is the only internal organ in constant contact with soil microorganisms [6, 10, 11].

The coelom, which grows in several groups of invertebrates, including earthworms, in the form of a large cavity, would be an ideal area for colonization by pathogens and parasites. The clearance of bacteria after experimental injection of bacteria into the coelomic fluid (CF) is usually complete within 48 h [12]. Therefore, this liquid could be considered aseptic. However, the CF of earthworms is in continuous contact with the external environment through excretory organs, nephridia and pores that exist on the external surface of the earthworm [12]. Thus, despite the immune property of this fluid, we couldn't neglect the possibility of a probable selection of microorganisms at this level. Hence, the main aim of this study is to perform a microbiological study by isolating culturable microorganisms, that could live in mutual association with the earthworm at the level of the immune system. The studied earthworm, which will be molecularly identified, is an endogenic type present abundantly in the temperate region of Bou Regreg-Akrach, Rabat-Morocco.

Knowing the continuous movement of earthworms, specially endogenies types, through the soil matrix, and considering the large population of beneficial plant growth rhizobacteria (PGPR) in the drilosphere and the rhizosphere, we are also interested in screening, qualitatively, bacteria with plant growth promoting potential.

Materials and Methods

Earthworm sampling

Earthworm sampling was carried out in Sidi Mohammed Ben Abdallah (SMBA) dam's surrounding soil, Bou Regreg-Akrach region, Rabat (Latitude: 33.95584800, Longitude: -6.72186300), Morocco. The dam of SMBA is located about 20 km south-east of Rabat-Salé, on Bou Regreg river.

Earthworms were collected in February 2019 during their peak period of activity in temperate climatic conditions (the average air temperature was close to 7°C) in order to avoid the period of dormancy. Soil pits (20 × 20 × 20 cm) were dug using a spade and earthworms were hand-sorted in the field. The earthworms collected were intended for morphological identification, as well as for molecular analysis and other experimental work. Specimens were first categorized into morphospecies based on their morphology, size, color and behavior. Adult earthworms with clitellum were weighed fresh (without emptying the intestinal contents), counted, and separated based on the identification key [13]. The abundant earthworm was selected for molecular identification. From this earthworm, the isolation of culturable microorganisms at the level of CF was carried out.

Molecular identification of earthworm

Molecular identification of earthworm was performed in Spain, Grupo de Ecología Animal (GEA), Universidade de Vigo, E-36310 Vigo. After washing earthworm with deionized water (D.I.W) and storing it in absolute ethanol, tissue samples were taken and the genomic DNA was extracted. Two universal invertebrate primers COI (LC01490 and HC02198; [14]) : COI F LCO1490 GGT-CAACAAATCATAAAGATATTGG and COI R HC02198 TAAACTTCAGGGTGACCAAAAAATCA, were used to amplify the DNA fragment encoding the cytochrome oxidase. The PCR reaction (40 µl) consisted of Master Mix (38 µl): H₂O PCR = 28.2 µl, Primer F (10 µM) = 2 µl, Primer R (10 µM) = 2 µl, Buffer Biotools (10x): Mg = 4 µl, dNTPs = 0.8 µl, Biotools Polymerase (1 U/ul) = 1 µl, and 2 µl of DNA template. The thermocycling protocol comprising an initial denaturation at 95°C (3 min), 40 cycles of denaturation at 94°C (30 sec), hybridization at 45°C (45 sec) and extension at 72°C (1 min), followed by a final extension at 72°C (5 min). Negative controls were

included to detect potential contamination. PCR products were sequenced in both directions using the Terminator Cycle Sequencing Kit following the protocol manufacturer's instructions.

Isolation of microorganisms from the coelomic fluid

The identified earthworm sample, from which the isolation was performed, were placed, aseptically, in a sterile bag and transferred to the laboratory within 4 h. The earthworm was washed with sterile distilled water to remove soil and roots. They were then fasted for 10 days in sterile glass petri dishes to avoid contamination by castings during isolation. Prior to puncture, the surface of an individual adult earthworm was disinfected by deceiving the worm in 1% Tween for 1 min. Then, the earthworm was washed in series four times with sterile deionised water and dried on sterile filter paper. A swab sample from the surface of the earthworm was taken, in order to check if some microorganisms could still attach to the surface after disinfection operation. The earthworm specimen was then placed in a sterile plastic bag to immobilize it and control its movements. A puncture at the lateral level of the posterior part of the earthworm was performed using a sterile glass capillary. The coelomic fluid of the earthworm was collected and homogenized in tube containing 3 ml of BHI (Brain Heart Infusion) liquid media. Each 0.1 ml of the homogenate was deposited on four different solid media:

- Brain Heart Infusion (BHI), - Medium prepared from earthworm's crude extract (ECEx), - Urea, - PDA (Potatoes Dextrose Agar) supplemented with chloramphenicol.

The crude extract (ECEx) was obtained by maceration in oven (60°C), executing successive baths of distilled water from a batch of dried earthworms. The residue, obtained after drying is consisting entirely of water-soluble matter. This residue constituted the crude extract of earthworms from which the ECEx culture medium was prepared (dry matter concentration: 0.25 g per 100 ml).

The prepared culture media were incubated at 30 and 25°C, respectively, for 7 days. Bacterial colonies and fungi were selected based on their morphological characteristics such as size, color and appearance. For the pure culture, the colonies were sub cultured and stored in a BHI liquid media supplemented with 20% glycerol, at

-20°C for later use.

Phenotypic characterization of isolates

The isolates were examined for colony and cell morphology, as well as for motility. Colony morphology was described using standard microbiological criteria, followed by Gram stain and microscopic observation of cells [15]. Biochemical tests were also performed using miniaturized and standardized galleries of biochemical tests: RapID remel (ThermoFisher Scientific).

Bacteria Identification: DNA extraction, 16S rDNA amplification and sequencing

The sequencing was carried out in the URATRS laboratory at CNRST Rabat, MOROCCO. Isolates with different phenotypic characteristics were selected. The bacterial DNA of each isolate was extracted from a pure bacterial culture according to the method provided by the BIOLINE "Isolate II Genomic DNA Kit" platform kit. The 16S rDNA of selected isolates was amplified, in order to obtain an amplicon of 1500 bp using the universal primers Fd1 and RP2, according to the following protocol: the reaction mixture contained (25 µl): 10 µl of sequencing buffer (2x Mix MyTaq from Bioline), 2 µl of the FD110 µM primer, 2 µl of the 10 µM RP2 primer, 130 ng of DNA template, and ultrapure water (for adjusting the volume to 25 µl). The PCR program used for amplification was as follows: 95°C, 1min; (95°C, 15 sec; 52°C, 20 sec; 72°C, 15 sec) 35 times; 72°C, 3min (Device: ABI "Verity" thermal cycler). Sequencing reactions were performed in 96-well PCR plates using the sequencing kit (Big Dye Terminator version 3.1 or version 1.1 cycle sequencing-Applied Biosystems).

Fungus identification: DNA extraction, ITS1 and ITS4 amplification and sequencing

The isolates were placed on potato dextrose agar (PDA) medium containing chloramphenicol (0.4 µg/l PDA). The plates were incubated at 25°C for 7 days to obtain a pure culture. A suspension containing the fungal spores was prepared in an ependorf. DNA from each isolate was extracted using the Genomic DNA Kit (Bioline). The DNA concentration obtained for each isolate was measured by Nanodrop. The amplification reaction was performed using the Kit MyTaq Extract-PCR 2X (Bioline), according to the manufacturer's

instructions. The amplified ITS were purified and eluted in sterile distilled water. Finally, the sequencing was carried out in the URATRS (Scientific Research Technical Support Units) laboratory at CNRST (The National Center for Scientific and Technical Research) Rabat, MOROCCO.

Phylogenetic analysis of DNA sequences

The sequence information was imported into the DNA Dragon program for assembly ("DNA Dragon: Sequence Contig DNA assembly software"). The assembled sequence of COI, also 16S rDNA and ITS rDNA from each isolate, were compared to that of type strains conserved in the GenBank database. Sequence similarity searches were performed using the BLAST program on the NCBI site. The strains with a high percentage of similarity to the isolate were then imported into MEGA X software so that their sequences were aligned [16]. Nucleotide substitution rates were calculated after alignment and phylogenetic trees were constructed using the "Neighbor-joining" method [17].

The GenBank accession number of the sequence reported in this paper are: MT878074, MT576611, MT576622, MT576620, MT576621, MT576623, MT576614, MT576615, MT576612, MT576624, MW282170, MT576613, MT576619, MT576616, MT576618, MT576610, MT576617, MW244587, MW244588.

Qualitative screening of bacteria with potential plant growth promoting traits

The identified isolates were subjected to qualitative tests, in order to demonstrate their possible potential to promote plant growth. The ability of isolates to bind nitrogen was revealed on Nitrogen free media Ashby [18]. The phosphate solubilizing potential was demonstrated on solid Nautiyal medium (modified PVK) [19]. Then, the solubilizing isolates of an insoluble form of potassium were selected on Alexandrov medium prepared on the basis of Feldspar powder [20]. Finally, the IAA test was performed in liquid medium of yeast extract Mannitol supplemented with 3 g/l of tryptophan according to the protocol of Shokri and Emtiazi [21]. On the basis of visual observation, the positive and negative test was recorded as + and –, respectively.

Results

Molecular identification of earthworm

The sampling of earthworms in the studied region (Bou Regreg- Akkrach, Morocco) showed the presence of three morphologically different genera of earthworms. The abundant earthworm was selected for molecular identification. From this earthworm, the isolation of culturable microorganisms at the level of the coelomic fluid (CF) was carried out.

Mitochondrial COI barcoding technique allowed us to identify the abundant earthworm in Bou Regreg-Akkarach region. Morphologic and phylogenetic studies showed that the COI sequence was much nearest to the specie *Aporrectodea molleri* (Accession number: MT878074) (Fig. 1).

Microbial community of the coelomic fluid

The structure of the culturable bacterial community of the CF of the newly identified earthworm was analysed. In total, 70 colonies grew on the 4 different culture media used (BHI, ECEx, Urea and PDA). The BHI culture medium allowed the growth of more bacterial colonies (34%), with different morphological appearance, followed by the ECEx medium (29%), and the Urea

Fig. 1. Phylogenetic trees obtained for the abundant earthworm of the region Akkrach (Rabat-Morocco), by sequencing the COI locus. Numbers at the nodes indicate percentage bootstrap values (%) support obtained in 1000 replications. The GenBank accession numbers for each sequence are shown adjacent to each strain. The scale represents 0.02 substitutions per site for COI segment.

Table 1. Number of *Aporrectodea molleri's* coelomic fluid isolates according to the isolation culture medium.

Culture media	Number of isolates (%)
BHI	24 (34%)
ECEx	20 (29%)
Urea	18 (26%)
PDA	8 (11%)

medium (26%) (Table 1). We noted that, most of the bacteria grown on the ECEx and BHI media also grew on the urea medium.

Phenotypic characterization of isolates

Based on the morphological appearance of colonies after subculturing and the results of fresh microscopic examination and Gram staining, catalase test and oxi-

dase test, 28 different bacterial isolates could be distinguished. All the 28 bacterial isolates were selected for identification by 16S rDNA molecular technique.

Two morphologically different fungi were selected for molecular identification based on sequencing ITS spacer region.

Microscopic examination as well as the biochemical tests were carried out on the molecular identified isolates as shown in the Tables 2 and 3.

All isolates were motile, catalase positive, and only one was cocci and 2 isolates were strict aerobic.

Approximately 81% (13/16) of the CF isolates were catalase positive, motile, aero-anaerobic bacilli that grew at temperatures between 28°C and 30°C. Out of 16, 10 isolates were Gram negative bacteria, which represent 63% (Table 2 and 3).

Most of gram-negative bacteria showed the ability to

Table 2. Phenotypic characteristics of the 10 Gram-negative bacteria isolated from the coelomic fluid of the earthworm *Aporrectodea molleri*.

Isolate		LC13	LC40	LC2	LC68	LC67	LC27	LC4	LC69	LC72	LC71
Optimal growth temperature		28 °C	28 °C	30 °C	30 °C	30 °C	28 °C	30 °C	30 °C	30 °C	30 °C
Respiratory mode ^a		Facult	Facult	Facult	Facult	Facult	Facult	Facult	Aero	Aero	Facult
Gram		-	-	-	-	-	-	-	-	-	-
Shape		bacille	bacille	bacille	bacille	bacille	bacille	bacille	bacille	bacille	bacille
Motility		+	+	+	+	+	+	+	+	+	+
Produced enzyme	Catalase	+	+	+	+	+	+	+	+	+	+
	Oxydase	+	+	-	-	-	-	-	+	+	+
	Arginine-dihydrolase	-	-	-	-	+	-	-	-	-	+
	Ornithine-decarboxylase	-	-	-	+	+	-	-	-	-	-
	Lysine-decarboxylase	-	-	+	+	-	-	+	-	-	-
	β-glucuronidase	-	-	-	-	-	-	-	-	-	-
	β-galactosidase	+	-	+/-	-	+/-	-	-	-	+/-	-
	β-glucosidase	+	+	+	+	-	+	+	-	-	-
	β-xylosidase	-	-	+	+	+	-	+	-	-	-
	β-glucosaminidase	+	+	+	+	+	-	-	-	+	-
	Proline-β-naphthylamidase	+	+	-	-	-	-	-	+/-	+/-	-
	Glutamyl-β-naphthylamidase	-	-	+	+	+	+	+	+	+	+
	Pyrrolidonyl-β-naphthylamidase	-	-	-	-	+	+	+	-	-	-
	Used substrate										
	Malonate	-	-	-	-	+	+	+	+	-	-
	Aliphatic thiol	-	+	-	+	-	+	-	+	+	-
	Fatty acid ester	+	+	-	-	-	-	-	-	-	+
	Sugar aldehyde	-	-	-	-	-	-	+	-	-	-
	Sorbitol	-	-	+	+	+	-	+	-	+	-

^aAero (Aerobic), Facult (Facultative), + (positive test) / - (negative test)

Table 3. Phenotypic characteristics of the 6 Gram-positive bacteria isolated from the coelomic fluid of the earthworm *Aporrectodea molleri*.

Isolate		LC47	LC12	LC15	LC9	LC31	LC46
Optimal growth temperature		30 °C	30 °C	30 °C	30 °C	30 °C	30 °C
Respiratory mode ^a		Facult	Facult	Facult	Facult	Facult	Facult
Gram		+	+	+	+	+	+
Shape		bacille	bacille	bacille	bacille	bacille	Cocci
Motility		+	+	+	+	+	+
Produced enzyme	Catalase	+	+	+	+	+	+
	Oxydase	-	-	-	-	-	-
	Arginine-dihydrolase	-	-	-	-	-	-
	Ornithine-decarboxylase	-	-	-	-	-	-
	Phosphatase	-	+	-	-	-	-
	α-glucosidase	+	+	-	-	-	-
	β-glucosidase	-	-	-	-	-	-
	β-galactosidase	-	-	-	-	-	-
	β-glucuronidase	-	-	-	-	-	-
	β-glucosaminidase	-	-	-	-	-	-
	Pyrrolidine aminopeptidase	-	-	-	-	-	-
	Arginine aminopeptidase	+	+	+	+	+	+
	Alanine aminopeptidase	+	+	+	+	+	+
	Leucine aminopeptidase	+	+	-	-	-	-
	Leucyl-glycine peptidase	-	-	-	-	-	-
Used substrate	Fatty acid ester	+	+	+	-	-	+
	Sucrose	+	+	-	-	-	+
	Mannose	+	+	-	-	-	+

^aAero (Aerobic), Facult (Facultative), + (positive test) / - (negative test)

produce β-glucosidase, β-glucosaminidase and glutamyl-β-naphthylamidase enzymes, and they can utilize substrate like aliphatic thiol and sorbitol. The enzymes produced by most Gram-positive bacteria were arginine aminopeptidase and alanine aminopeptidase, and the substrate was the fatty acid ester (Table 2 and 3).

Molecular identification of bacterial isolates

To identify CF isolates (Bacteria and fungi) at the species level, molecular phylogenetic trees were constructed based on 16S rDNA and ITS sequences, using the Neighbor-Joining method for evolutionary distances. The results of the phylogenetic study of the CF bacteria and fungi were shown in Figs. 2 and Fig. 3, respectively. The results showed that the 28 bacterial isolates were phylogenetically close to 16 different bacterial species, which belong to 5 different bacterial families.

Among the isolated bacteria, 31% (5/16) belong to the *Bacilliaceae* family, 31% (5/16) to the *Enterobacteriaceae* family, 19% (3/16) to *Pseudomonaceae*, 13% to *Aeromonaceae* (2/16) and 6% to *Staphylococcaceae* (1/16).

Among the isolated bacteria, 37% belong to Gram positive bacteria distributed as follows: 31% (5/16) belong to the *Bacilliaceae* family and 6% (1/16) to *Staphylococcaceae*. The rest of the isolates 63% (10/16) were Gram negative represented by 3 families: *Enterobacteriaceae*, 31% (5/16), *Pseudomonaceae*, 19% (3/16) and *Aeromonaceae* 13% (2/16).

Generally, the similarities sequences of the isolates were greater than 97.58% (cut off) to their relative reference strains in the GenBank database (Table 4).

Five bacterial isolates belonging to *Enterobacteriaceae* family were phylogenetically closers to *Raoultella planticola* ATCC 33531 (LC4 (1522 nt)), *Klebsiella aerogenes*

Fig. 2. Phylogenetic tree showing the position of the strains isolated from the coelomic fluid of earthworm *Aporectodea mollerii*. The evolutionary distances were computed using bootstrap test (1500 replicates) Kimura 2-parameter.

NBRC 13534 (LC67 (1518 nt)), *Buttiauxella gaviniae* S1 /1-984 (LC2 and LC68 (1508 and 1509 nt)) and *Pantoea vagans* LMG 24199 (LC27 (1514 nt)), with a similarity percentage of 98.52%, 98.91%, 98.39, 99.26% and 98.87, respectively (Fig. 2, Table 4). Despite that the isolates LC2 and LC68 are close to the same cluster, they are phylogenetically different strains.

The bacterial isolates LC40 and LC13 belong to *Aeromonas* genus (Fig. 2). The genetic sequence of 16S rDNA from strain LC40 (1530 nt) did not represent a close phylogenetic similarity with the 16S rDNA gene sequences recorded in the Genbank Database. The 16S rDNA gene sequence of LC13 (1529 nt) was phylogenetically closest to the 16S rDNA gene sequences of *Aeromonas hydrophila* JCM 1027 with a sequence similarity of 99.73%.

The genus of *Pseudomonas* was divided into 3 phylogenetically different species (Fig. 2). LC71 (1505 nt), LC69 (1535 nt), and LC72 (1517 nt) had the closest similarities at a percentage of 99.11%, 99.45% and 98.71%, with *Pseudomonas aeruginosa* strain NBRC 12689, *Pseudomonas azotoformans* NBRC 12693 and *Pseudomonas helmanticensis* strain OHA11, respectively (Fig. 2, Table 4).

From the CF, it was possible to isolate a strain belonging to the *Staphylococcaceae* family. Isolate LC46 was phylogenetically close to the 16S rDNA gene sequences of *Staphylococcus epidermidis* NBRC 100911, with a sequence similarity of 97.58% (Fig. 2, Table 4).

Bacillus genus included five bacterial species. LC47 (1517 nt), LC12 (1201 nt), LC9 (1529 nt), LC31 (1536 nt) and LC15 (1205 nt) were closer to *Bacillus paramycoides* MCCC 1A04098, *Bacillus thuringiensis* IAM 12077, *Bacillus badius* NBRC 15713, *Bacillus licheniformis* BCRC 11702, and *Bacillus altitudinis* 41KF2b with rates of 16S rDNA sequence similarity of 99.93%, 99.6%, 99.6%, 98.25% and 100%, respectively (Fig. 2 and Table 4).

Molecular identification of fungi isolates

Based on ITS1 and ITS4 sequences, the fungi LC44 and LC51 showed a phylogenetic similarity to the species *Penicillium griseofulvum* CBS 185.27 and *Penicillium polonicum* CBS 222.28, with 100% and 98.87% percentage of identity, respectively (Fig. 3 and Table 4).

Table 4. Sequence similarities of 16 bacteria and 2 fungi isolated from the coelomic fluid of the earthworm *Aporrectodea molleri*, to closest relatives.

Strains	Nearest Species	Query length (nt)	Identity (%)	Accession Number	Number of nucleotides (nt)
LC4	<i>Raoultella planticola</i> ATCC 33531 ^T	93%	98.52%	MT576611	1522
LC67	<i>Klebsiella aerogenes</i> NBRC 13534 ^T	96%	98.91%	MT576622	1518
LC2	<i>Buttiauxella gaviniae</i> S1/1-984 ^T	98%	98.39%	MT576620	1508
LC68	<i>Buttiauxella gaviniae</i> S1/1-984 ^T	98%	99.26%	MT576621	1509
LC27	<i>Pantoea vagans</i> LMG 24199 ^T	93%	98.87%	MT576623	1514
LC40	<i>Aeromonas</i> spp	-	-	MT576614	1530
LC13	<i>Aeromonas hydrophila</i> JCM1027 ^T	96%	99.73%	MT576615	1529
LC 71	<i>Pseudomonas aeruginosa</i> NBRC 12689 ^T	97%	99.11%	MT576612	1505
LC69	<i>Pseudomonas azotoformans</i> NBRC 12693 ^T	96%	99.45%	MT576624	1535
LC72	<i>Pseudomonas helmanticensis</i> OHA 11 ^T	97%	98.71%	MW282170	1517
LC46	<i>Staphylococcus epidermidis</i> NBRC 100911 ^T	96%	97.58%	MT576613	1534
LC47	<i>Bacillus paramycoides</i> MCCC 1A04098 ^T	99%	99.93%	MT576619	1517
LC12	<i>Bacillus thuringiensis</i> IAM 12077 ^T	97%	99.6%	MT576616	1201
LC9	<i>Bacillus badius</i> NBRC 15713 ^T	96%	99.6%	MT576618	1529
LC31	<i>Bacillus licheniformis</i> BCRC 11702 ^T	96%	98.25%	MT576610	1536
LC15	<i>Bacillus altitudinis</i> 41KF2b ^T	96%	100%	MT576617	1205
LC 44	<i>Penicillium griseofulvum</i> CBS 185.27 ^T	100%	100%	MW244587	188
LC51	<i>Penicillium polonicum</i> CBS 222.28 ^T	93%	98.87%	MW244588	188

Fig. 3. Phylogenetic tree showing the position of the fungus isolated from the coelomic fluid of earthworm *Aporrectodea molleri*. The evolutionary distances were computed using bootstrap test (1500 replicates) Kimura 2-parameter.**Earthworm's cutaneous surface isolates**

The bacteria isolated from the surface of earthworm

Fig. 4. Phylogenetic tree showing the position of the strains isolated from the surface of earthworm after disinfection: ECSB1 and ECSB2. The evolutionary distances were computed using bootstrap test (1500 replicates) Kimura 2-parameter.

and those resistant to the disinfection treatment, were also intended for molecular identification by amplification and sequencing of the 16S rDNA gene. The phylogenetic analysis showed that earthworm's cutaneous surface bacteria (ECSB) were different from those of the CF. Cutaneous Surface bacteria ECSB1 and ECSB2

were phylogenetically nearest to *Pseudomonas reinekei* MT1 and *Staphylococcus petrasii* CCM 8418, respectively (Fig. 4).

Qualitative screening for PGP ability of isolated bacteria

Table 5 represents the results of tests carried out in solid medium for the qualitative revelation of bacteria that have the ability of fixing nitrogen, solubilizing phosphate and potassium and those capable of producing the phytohormone IAA. Out of 16 tested bacteria, nine can fix atmospheric nitrogen while growing in Ashby media. The qualitative test on the solid PVK media showed that, four isolates formed a halo around the colonies, which means they can solubilize the insoluble form of phosphate Tricalcium phosphate. Six bacteria had the potential to solubilize insoluble form of potassium (Feldspar) in Alexandrov solid media. The qualitative test in liquid media supplied by Tryptophane, showed that among the 16 bacterial isolates of the CF, eleven isolates can produce indole acetic acid (69%). The isolated bacteria LC68, LC27 and LC71 showed a positive performance for the all 4 tests. These bacteria belong to *Buttiauxella*, *Pantoea* and *Pseudomonas* genera, respec-

tively.

We notice that 10/16 isolates are positive for at least 2 tests and only 3/16 isolates (LC67, LC2 and LC72) are negative for the 4 tests.

Also, although the 2 isolates, LC2 and LC68 were phylogenetically close to the same species, they do not have the same potential. LC2 did not show any potential concerning the plant growth promoting traits.

Discussion

Our study aimed to demonstrate the existence of a symbiotic association between an abundant endogenic earthworm, inhabiting the temperate zone of Sidi Mohamed Ben Abdellah dam SMBA (Bou Regreg-Akrech, Morocco) and microorganisms living in the earthworm's immune system; the coelomic fluid (CF).

The abundant earthworm of the SMBA region belongs to the genera *Aporrectodea*. Unlike *Aporrectodea caliginosa* species, which has known effects in soil as priming the release of mobile and available micronutrients, *Aporrectodea molleri* specie is still not well studied concerning its role in the soil [23].

Table 5. Qualitative screenings of bacteria with the potential to fixe nitrogen, solubilize tricalcium phosphate and potassium, and produce IAA.

Family	Strains	Nitrogen fixation	Phosphate solubilization	Potassium solubilization	IAA production
<i>Enterobacteriaceae</i>	LC4	+	-	+	-
	LC67	-	-	-	-
	LC2	-	-	-	-
	LC68	+	+	+	+
	LC27	+	+	+	+
<i>Aeromonadaceae</i>	LC40	+	-	+	+
	LC13	-	-	+	+
<i>Pseudomonadaceae</i>	LC 71	+	+	+	+
	LC69	+	-	-	+
	LC72	-	-	-	-
<i>Staphylococcaceae</i>	LC46	+	+	-	-
<i>Bacillaceae</i>	LC47	+	-	-	+
	LC12	+	-	-	+
	LC9	-	-	-	+
	LC31	-	-	-	+
	LC15	-	-	-	+

+ (positive test) / - (negative test)

The medium prepared from the freshly harvested earthworm extract (ECEx) allowed the growth of colonies of different sizes and appearances, as BHI medium. This result confirms that the medium prepared from whole earthworms is as rich as the BHI medium and allows the growth and isolation of bacteria that belong to different genera. This can be explained by studies which have shown that earthworms are very rich in protein (from 32.6% to 67.2%), amino acids, fats (7 to 10%), carbohydrates (8 to 20%), minerals and various vitamins (2 to 3%) [24–26]. Therefore, earthworm would constitute a favorable environment for the growth of exogenous bacteria and saprophytes fungi if it were not protected by an effective immune system.

The effectiveness of antimicrobial activity of earthworm's CF has been tested in a fair number of scientific researches [27–29]. Innate immunity is maintained by cellular components and numerous active immunological (antimicrobial) molecules [30]. Antimicrobial peptides represent a firstline innate immune response. One of the widely distributed antimicrobial peptides is lysozyme. This protein cleaves the β -1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan of bacterial cell walls and thus effectively contributes to the protection against infections caused mainly by Gram-positive bacteria [31]. However, it has been found that the CF of earthworms contains molecules exert antimicrobial activity against both Gram-positive and Gram-negative bacteria, but, in particular against strains pathogenic for earthworms [32, 33]. In the present study, Gram positive bacteria represented only 38% of total isolated bacteria. This let us suggest that the selection can be based either on membrane constitutions or a species-specific interaction. The very low number of fungi isolated from the CF can be explained by the fact that generally earthworms have a negative effect on fungus except some species. It has been suggested that some fungal species appear to be preferred while others are rejected, but the extent to which other earthworm species (particularly endogenic) select different fungal species is not well established. Moreover, we have to note that earthworms increase bacterial/ fungal ratio in the soil [8]. The fact that some fungi are selectively rejected by earthworms, can be related to their ability to affect the survival and growth of earthworms by producing toxins [28].

The presence of two bacteria belonging to staphylococcus genera in the surface of earthworm, reflect their resistance to disinfection treatment, or the protection by surface mucilage. Nevertheless, the cutaneous mucus of earthworm consists of several antimicrobial agents that provide a first line of defence against invading pathogens, but in the same time it is selective. Indeed, bacteriostasis of earthworm secretions increased significantly after exposure to *E. coli* and *S. aureus*, although no bacteriostatic effect was observed for *P. aeruginosa* [34].

The molecular analysis of CF's isolated bacteria, revealed the existence of 16 bacterial species belonging to 5 different families, namely: *Aeromonaceae*, *Bacillaceae*, *Pseudomonaceae*, *Enterobacteriaceae* and *Staphylococcaceae*.

The present microbiological study firmed that CF, which fills the coelomic cavity, is not aseptic. In fact, each segment of the earthworm cavity interfaces with the external environment via excretory organs and dorsal pores which allow microorganisms to enter CF [12]. Therefore, CF interacts with soil microorganisms. The microorganisms which cross the epithelial barrier in CF are eliminated by the efficient innate defence mechanisms, just mentioned, which are provided by the coelomocytes and by various humoral antimicrobial factors involved in the direct elimination of invading bacteria. Yet, there may be a mutual symbiotic association which has caused some bacteria to survive under these conditions and which are not affected by the antimicrobial substances in this system. For the species of the earthworm *A. molleri*, from which the isolation was made, it is assumed that the 16 isolated bacterial species were selected by the earthworm and they live in symbiotic association at the level of its CF. Since earthworms live in the rhizosphere and feed on plant roots as organic matter, they promote the spread and growth of beneficial soil bacteria.

We mention that, among the isolated and identified species of CF, there are species which their important role in the rhizosphere has been proved and which are qualified as beneficial bacteria for plant growth. *Bacillus thuringiensis*, *Bacillus altitudinis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Pseudomonas azotiformans*, *Pseudomonas helmanticensis*, have effects on stimulating plant growth, and used in biocontrol and soil fertilization [35–39].

If we analyse the results of bacteria isolated from the intestine of earthworms, it can be noted that *Aeromonas*, *Bacillus* and *Pseudomonas* are the most dominant genera in different species of earthworms. Picón *et al.* [6] revealed the presence of a variety of bacterial strains in the intestine of earthworms (*Eisenia andrei*), among which 8 species, belonging to *Pseudomonadaceae*, *Bacillaceae* and *Enterobacteriaceae* families, have been identified. In addition, Kim *et al.* [10] have proved the presence of *Aeromonas*, *Enterobacter* and *Bacillus* genera, with *Bacillus* the most abundant genus, in the earthworm's gut.

Recent studies have proved the effect of bacteria associated to earthworm on the remediation and plant growth. Biswas *et al.* [40] have isolated three phosphate solubilizing bacteria (*Bacillus megaterium*, *Staphylococcus haemolyticus* and *Bacillus licheniformis*) from the gut of the earthworm *Metaphire posthuma*. In the study, it has been suggested that the three isolated phosphate solubilizing bacteria strains from earthworm gut possess intrinsic abilities of growth promotion, metal resistance, which could be exploited for plant growth promotion and bioremediation even under metal-stress conditions.

Based on our study it can be assumed that that earthworm, during their peristaltic movements, they are involved in the dynamics and the dispersion in the soil of beneficial bacteria, through the release of CF. This interaction is essential in the natural improvement of soil fertility.

To well understand the role of CF microorganisms, ulterior studies should be conducted and the role of CF-associated microorganisms should be tested in vivo for plant growth.

Acknowledgments

The work is carried out with the support of the National Center for Scientific and Technical Research (CNRST) within the framework of the Research Excellence Scholarships Program.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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