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Original article

Characterization of possible symbionts in *Onychochaeta borincana* (Annelida: Glossoscolecidae)

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Abstract

The presence of symbionts in the gut of *Onychochaeta borincana* has been suggested but they have never been characterized. The intestinal tissue of this species was examined using electron microscopy and microbiological and molecular biology techniques in order to identify the possible gut associated symbiont. Four gram-positive, endospore-forming and β-hemolytic bacterial strains remained adhered to the intestinal wall of these organisms even after intense cleaning. Molecular and physiological characterization proved their similarity with *Bacillus* genus, which is typically found in soils and has been reported as a possible intestinal symbiont in arthropods. These results were corroborated by SEM, which showed bacilli structures adhered to the intestinal wall. However, results of molecular tests indicate that a low number of microorganisms remained adhered to intestinal wall after intense cleaning.

Keywords: Intestine; Symbiotic; Earthworm; Bacteria

1. Introduction

Onychochaeta borincana Borges, 1994 is a glossoscolecid reported only from Puerto Rico. It is easily identified by the presence of hook-shaped setae in the posterior part of the body, a characteristic that no other species on the island has. This earthworm is relatively common in Puerto Rico, especially in disturbed areas [3], and has also been found in soils with very low organic matter content (1.34%) [1], a condition that limits the survival of many endogeic earthworms. This fact suggests the possibility of a symbiotic microorganism aiding in its digestive process. Several studies have been performed in order to assess this prospect [2,9]. Using scanning electron microscopy (SEM), Méndez et al. [8] observed segmented filamentous bacterial (SFB) adhered to the intestinal wall of *O. borincana* by means of a "socket-like" structure similar to the one reported in other earthworm species [6]. Méndez et al. [8] suggest that these SFB could be gut-associated symbionts, but to date the SFB in *O. borincana* have not been identified.

The aim of this study was to identify the potential symbiont adhered to the gut wall of *O. borincana* using SEM, microbiological culture techniques, polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH).

2. Materials and methods

2.1. Specimen preparation

Twenty adult specimens were extracted from clayish soil in a wooded area located in the environs of the

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Biology Building of the University of Puerto Rico at Mayagüez (western part of Puerto Rico). The specimens were placed in sterilized soil (3 autoclave cycles at 121 °C and 15 psi for 15 min) for 24 h. They were immersed in hot water (60–70 °C) for 20 s and then dissected through their mid-ventral line to remove their guts, which were cleaned intensely [10]. Briefly, the intestine was placed in a 1.5 ml Eppendorf tube with 1.0 ml of phosphate buffer, 0.1 M at pH 7.2; and vortexed for 30 s. The intestines were transferred into fresh buffer solution and the washes were repeated five times.

2.2. Microbiological tests

Twelve earthworm guts were opened longitudinally and their inner walls were placed in direct contact with Petri plates containing four different culture media (Luria Bertani agar, Nutrient agar, and Actinomycetes isolation, all of Difco®, and Casein agar [12]). Trials were done in triplicate and then incubated at 25, 32, and 37 °C. Microbial growth was monitored every 12 h for 3 days, and the colony forming units per gut (cfu/gut) were determined. The isolated bacterial strains were analyzed microbiologically at macroscopic and microscopic levels by determining the colony and cell morphology, reaction to Gram stain, and the presence of capsule and endospore formation as described by Cappuccino et al. [4]. Also, biochemical and physiological analyses, such as hemolysis test and the physiological utilization of carbon sources using BIOLOG®, were performed for each strain.

2.3. SEM

Two guts were fixed in 4% glutaraldehyde in phosphate buffer saline (PBS), 0.1 M at pH 7.2, for 24 h. Their guts were extracted, cleaned and processed for SEM according to Méndez et al. [8]. The bacterial cultures isolated through this study were also processed following the Vega et al. protocol (personal communication in April 2006) and viewed by SEM.

2.4. DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA (gDNA) was extracted from four earthworm guts using a FastDNA® Kit (Q-biogen) following the manufacturer's specifications. Humic acids were removed from the DNA using a Sephadex G50 chromatography column [12]. A 0.8% agarose gel electrophoresis was used to confirm the gDNA extraction. gDNA was also extracted from the strains isolated from the microbiological probe. PCR reaction of 16S rDNA was performed as described by Furlong et al. [5]. Four other intestines were intensely cleaned and mixed with 150 μl of an overnight bacterial culture in broth. Next, the mixture was processed for total gDNA extraction, and used as positive control to detect the presence of any inhibitor agent in the gDNA. PCR results were sent to be sequenced at Macrogen Company USA (http://www.macrogenusa.com/com/). The sequencing results were analyzed *in silico* using DNA analysis, programs available on line, such as BLAST (http://www.ncbi.nlm.nih.gov/blast/). For the phylogenetic analyses, the sequences were aligned using the CLUSTAL W program, and the phylogeny was constructed with Jukes–Cantor distance matrices for inferring the tree topology and neighbor joining and maximum-parsimony for bootstrap analysis (1000 replicates) of the branching pattern using the MEGA 3.1 software.

2.5. Fluorescence in situ hybridization

Scanning electron microscopy showed that a large number of bacteria remained in the guts of *O. borincana* after they were washed moderately [8] (data not shown). For this reason moderately washed intestines were used as positive control.

Intensely [10] and moderately washed intestines were examined by FISH using the EUB 338 probe with Cy3™ and Cy5™ following Snaidr et al. [11], and 0.85% NaCl as washing buffer. Hybridization was carried out in a 15 ml Falcon® tube and the stained samples were spread on glass slides and allowed to air dry prior to observation. Earthworm tissue and associated bacteria were observed using a confocal laser scanning microscope Fluoview FV 300. Nomarsky and fluorescence images were overlapped.

3. Results

3.1. Microbiological tests

Four different bacterial strains were isolated from all the culture media used in this study. The isolates showed similar microscopic morphology (gram-positives rods and endospore-forming) and growth (approximately 1.2×10^7 cfu/gut) characteristics. Physiological analysis using BIOLOG® and hemolysis tests suggest that the four strains belong to the *Bacillus* genus and that two of these strains are similar to *Bacillus* genus (Table 1). The individual cell size range from all isolated bacteria showed similar dimensions (0.2–0.4 μm