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Identification and localization of food-source microbial nucleic acids inside soil nematodes

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ABSTRACT

Microorganisms (e.g., prokaryotes, fungi) are food sources for soil nematodes, but they can also be potential mutualists or pathogens. Understanding the linkages between microorganism and invertebrate diversity in soils requires the ability to distinguish between these microbial roles. We tested the potential of a taxon-specific fluorescent *in situ* hybridization (FISH) procedure for identifying and localizing microbial rRNA within the bodies of soil nematodes. Our objective was to determine whether the rate of digestion permitted detection and identification of food-source nucleic acids within the nematode digestive system (i.e., pharynges, intestines) before their breakdown. First, using laboratory cultures of *Caenorhabditis elegans* maintained on *Escherichia coli*, we were able to localize bacterial rRNA throughout the nematode pharynx with the universal bacterial-probe EUB338, although never in the intestines. Second, we applied the fungal rRNA probe FR1 to *Aphelenchus avenae* cultured on the fungus *Rhizoctonia solani*. We were unable to detect fungal rRNA within these nematodes, and it appears that this material may be digested rapidly. Next, we applied our technique to nematodes extracted directly from soils. We were able to localize bacterial rRNA within the pharynges of bacterial-feeding species of nematodes from desert soils. We also localized archaeal rRNA using the probe ARC344. Finally, application of EUB338 to desert soil nematodes revealed the presence of bacteria in the intestines of some nematodes and within the ovary of a single nematode. This technique has great potential for use in understanding the feeding behavior of bacterial-feeding soil nematodes and in studies of nematode:bacterial relationships.

1. Introduction

Nematodes are the most abundant and widespread form of animal life on Earth (Bongers and Ferris, 1999). In soils, nematodes contribute to the delivery of nutrients to growing plants, to carbon cycling, and to soil formation (van der Putten et al., 2004). Soil nematode communities are species-rich and functionally diverse and include species that can feed on plant roots, bacteria, fungi, protozoa, and other nematodes and soil invertebrates, either exclusively or as omnivores (Yeates et al., 1993). Despite the importance of nematodes in ecosystems, gaps remain in our understanding of the ecology of these primarily microscopic organisms, particularly regarding their trophic behavior (Ferris and Bongers, 2006; Yeates et al., 1993).

Traditionally, nematode species have been assigned to trophic groups (bacterial-feeders, fungal-feeders, plant-feeders, etc.) based on the type of mouth parts they possess (Yeates et al., 1993). For example, plant-parasitic, fungal-feeding, and many omnivorous species bear various types of protrusible stylets. These hollow tubes are used to pierce the walls of plant or fungal cells for suction of cell contents into the nematode pharynx. Bacterial-feeding nematodes have several distinct mouth-part morphologies, with many species bearing labial and cephalic projections. These structures are believed to play either a sensory role in detecting food in the environment and/or a mechanical role in scraping or sorting bacterial food items and propelling cells into the mouth cavity (De Ley, 1992).

The link between morphology and trophic behavior has been supported for many nematode species in laboratory cultures, and these results have been extended broadly to uncultured species based on shared morphology (Yeates et al., 1993). Unfortunately, the hypothesis that feeding is related to morphology cannot be confirmed by direct observation of nematode behavior in soils and sediments. Therefore, there is some ambiguity associated with the assignment of many nematode species to specific trophic groups (Neher, 2001; Yeates et al., 1993). For example, the family Tylenchidae contains species with small stylets (i.e., compared to the larger stylets of confirmed plant-parasitic nematodes). Nematologists have been unable to determine whether species of Tylenchidae graze on plant root hairs, fungal hyphae, or both (Yeates et al., 1993). Furthermore, it is not known whether nematodes

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have feeding preferences *within* their assigned trophic group (e.g., do bacterial-feeders consume all species of prokaryotes that they encounter or do they feed exclusively on specific types?).

Until recently, there have not been any acceptable culture-independent methods for determining nematode feeding preferences. Insect ecologists have used PCR-based analyses successfully to identify the digestive system contents of their study organisms (King et al., 2008). This technique also has been used to identify the nematode species being consumed by predatory nematodes (Neilson et al., 2006). Ladygina et al. (2009) used partial 16S rDNA gene sequences PCR-amplified from nematodes to examine the diversity of bacteria associated with nematodes from different trophic groups. However, PCR-based approaches that attempt to identify what a nematode has consumed cannot distinguish food DNA from DNA that may be adhering to the nematode surface due to acquisition from its habitat. Unfortunately, the efficacy of nematode surface sterilization techniques toward the removal of microbial nucleic acids has not been established. Furthermore, little is known about the microbial endosymbionts that nematodes harbor, although many have been observed (Haegeman et al., 2009; Musat et al., 2007; Snyder et al., 2007; Vandekerckhove et al., 2000). Because PCR-based techniques do not reveal the source of bacterial, archaeal, or fungal DNA amplified from a nematode, it is difficult to determine whether recovered sequences represent surface contamination, pathogens, symbionts, or consumed food. Therefore, PCR-based approaches cannot be easily used to resolve the feeding preferences of soil nematodes.

Fluorescent *in situ* hybridization (FISH) with taxon-specific oligonucleotide probes has proved to be a viable alternative to PCR for identifying microbes *in situ* (Amann et al., 1995). Here, we report our efforts to use FISH to identify and localize food-source nucleic acids (bacterial, archaeal, and fungal small subunit ribosomal RNA) within nematodes, post-consumption. We used a suite of oligonucleotide probes that hybridize to highly conserved sequences within target food sources. Our main objective was to determine whether food-source nucleic acids were detectable within nematode bodies or whether they were too rapidly digested. We used nematode species (*Caenorhabditis elegans*, *Aphelenchus avenae*) cultured on single, defined food sources (fungi or bacteria) to test this technique, and we predicted that we would be able to localize microbial rRNA in the nematode pharynges or intestines. Once the efficacy of this technique was established, our second objective was to detect the microbial contents of nematodes extracted directly from soils, where they had been feeding *in situ*. These experiments used two groups of soil nematodes: (1) stylet-bearing Tylenchidae (putative fungal-feeders) from forest soils and (2) bacterial-feeding nematodes from desert soils.

2. Materials and methods

2.1. Fluorescent in situ hybridization procedure

A FISH procedure for nematodes was modified from Vandekerckhove et al. (2002). Nematodes were prepared for hybridization in 0.5 ml microcentrifuge tubes equipped with 0.65 μm filters (Ultrafree MC, Millipore, Billerica, MA, USA). The filters allowed for application of solutions and their subsequent removal via centrifugation, without loss of nematodes. Nematodes were transferred to tubes (10–30 nematodes tube^{-1}) and centrifuged briefly (2000 × g) to remove excess transfer liquid. Prior to application of oligonucleotide probes, nematodes were processed through a series of steps for surface cleansing and fixation/permeabilization of their tissues. First, the nematodes were immersed in 0.1% benzalkonium chloride (1 min) followed by two rinses in 0.85% sodium chloride (2 min each). Second, fixation was performed in a 1:1 mixture of glacial acetic acid and ethanol (10 min) followed by two rinses in pure ethanol (5 min each). Next, nematodes were rinsed (10 min) in 1:1 methanol and phosphate-buffered Tween (PBT; 150 mM NaCl, 10 mM Na_3PO_4, 0.1% Tween 20, pH 7.4). Then, the nematodes were washed with 1.0% formaldehyde in PBT (30 min), followed by two rinses in PBT (2 min each). After each step, the tubes were centrifuged briefly to force the solution through the filter for disposal.

For the hybridization steps, nematodes were transferred from the filter surface to a new microcentrifuge tube (1.5 ml, filterless) via three 100 μl aliquots of hybridization mixture (20 mM Tris–HCl, 0.02% SDS, 0.9 M NaCl, 5 mM EDTA, 60% formamide, pH 7.4). The three washes maximized the proportion of nematodes that were transferred, but a proportion of the original number was still lost at this or at subsequent transfer steps. Herring sperm DNA was also added to the hybridization mixture (final concentration 2 μg ml^{-1}) to hinder non-specific binding of the probe to the nematodes. A final aliquot of hybridization mixture (100 μl) containing the oligonucleotide probe (Table 1, Invitrogen, Carlsbad, CA, USA) was added to the transferred nematodes for a final probe concentration of 1 μM. The tubes were wrapped with foil and placed in a darkened incubator at the appropriate high stringency hybridization temperature (Table 1) for 3–48 h. Following this incubation, samples were rinsed twice (30 min each) at 48 °C (55 °C for ARC344) in hybridization buffer (20 mM Tris–HCl, 0.02% SDS, 0.008 M NaCl, 5 mM EDTA, pH 7.4). In between each wash step, the tubes were centrifuged briefly to limit removal of nematodes with removal of wash solutions.

Following the last hybridization buffer wash and centrifugation, the supernatant was removed, and the nematodes were re-suspended in 30 μl 2% (w/v) DABCO (1,4-diazobicylco[2.2.2]octane) in 40% (v/v) glycerol in PBS to preserve fluorescence. This solution was transferred onto microscope slides and covered with a cover slip. Drops of nail polish were applied to the slides to raise the cover slips slightly and prevent flattening of the nematodes. Cover slips were sealed to the slides with a layer of nail polish. Slides were stored at 5 °C in the dark until the samples could be examined microscopically.

Smears were prepared on slides from cultures of bacteria (*Escherichia coli* OP50), archaea (*Halobacterium* sp. NRC-1, Carolina Biological Supply Co., Burlington, NC, USA), or fungi (*Rhizoctonia solani*), heat fixed, and used as positive controls for hybridization

Table 1
Oligonucleotide probes.[a]

Probe name	Sequence (5′ → 3′)	Target taxa	Hybridization temperature (°C)	Reference
EUK516	ACCAG ATTGC CCTCC	Eukarya	40	Amann et al. (1990)
NON338	ACTCC TACGG GAGGC AGC	Negative control	46	Wallner et al. (1993)
EUB338	GCTGC CTCCC GTAGG AGT	Bacteria	46	Amann et al. (1990)
ARC344	TCGCG CCTGC TGCIC CCCGT	Archaea	53	Raskin et al. (1994)
FR1	CTCTC AATCT GTCAA TCCTT ATT	Fungi	40	Hagn et al. (2003), Zhou et al. (2000)

[a] Oligonucleotide probes were labeled at their 5′ end with fluorescein (green) or AlexaFluor 546 (red; Invitrogen, Carlsbad, CA, USA). Probes were rehydrated (10 μM) in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4).