

A new and simple technique for the isolation of symbiotic bacteria associated with entomopathogenic nematodes (Heterorhabditidae and Steinernematidae)

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Abstract: This study describes a new, simple, and economical method for isolation of symbiotic bacteria from entomopathogenic nematodes. Isolation of the symbiotic bacteria from entomopathogenic nematodes is useful for mass production of the nematodes. In addition to traditional isolation methods from infected insect larvae or infective juveniles, we show a new method of isolating symbiotic bacteria from either gravid females or females in the *endotokia matricida* stage, producing symbiont colonies after a very short period of surface sterilization.

Key words: *Heterorhabditis*, *Steinernema*, symbiotic bacteria, *Photorhabdus*, *Xenorhabdus*

The entomopathogenic nematode species (EPNs) of 2 genera, *Heterorhabditis* and *Steinernema*, are effective biological control agents against major insect pests (Kaya and Gaugler, 1993). These nematodes each contain symbiotic bacteria, with *Photorhabdus* spp. for *Heterorhabditis* and *Xenorhabdus* spp. for *Steinernema* (Akhurst, 1980; Boemare et al., 1993). The symbiotic bacteria reside in the nematode intestine (for *Heterorhabditis*) or in a bacterial vesicle (for *Steinernema*) and are released into the insect hemocoel when the nematode enters the target insect host (Dowds and Peters, 2002). The bacteria multiply, producing septicemia, and kill the insect host within 24 to 48 h, allowing the colonizing nematodes to feed on both the bacteria and the digested insect tissues (Dunphy and Webster, 1988; Park and Kim, 2000). Once developed, infective juveniles (IJs) emerge from the cadaver and search for another host.

These bacteria are of interest not only for the mutualistic association with EPNs, but also for their production of toxins, antibiotics, and enzymes (Webster et al., 2002; Park and Forst, 2006; Proschak et al., 2011). As there are currently no selective media for these bacteria and free-living forms of these bacteria have not yet been discovered, to carry out research on these bacteria, they must first be isolated from their nematode hosts.

Three methods have been used for isolating symbiotic bacteria from entomopathogenic nematodes. The "hanging drop" method uses a sterile drop of insect hemolymph,

to which surface-disinfected IJs are added (Poinar and Thomas, 1966). The nematodes exsheath their old cuticles in the hemolymph droplet and commence development, releasing their symbiont into the hemolymph, where the bacteria grow and can be subcultured after about 24 h. The second method is to collect a drop of insect hemolymph under sterile conditions from an insect 24 h after infection and to streak it onto nutrient agar. The third method involves crushing about 100 surface-disinfected IJs and streaking the macerate onto nutrient agar (Akhurst, 1980). However, all of these methods produce mixed cultures of bacteria, including contaminating bacteria from both the insect and the nematode.

In a study of the *P. luminescens*-*H. bacteriophora* symbiosis, Ciche et al. (2008) showed that the number of bacterial symbionts reached a maximum in the *endotokia matricida* stage, when the mature female is filled with developing larvae prior to emergence, which causes matricide (Johnigk and Ehlers, 1999). This suggested that it might be possible to improve the efficiency of bacterial isolation by targeting the mature female stages of nematode; thus, studies were carried out to isolate the bacterial symbionts from both heterorhabditid and steinernematid species.

Greater wax moth (*Galleria mellonella*) larvae were used to obtain mature female and hermaphrodite nematodes. *G. mellonella* larvae were infected with *Steinernema feltiae* (Turkish isolate 09-38) and *Heterorhabditis bacteriophora*

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(Turkish isolate 09-43) by placing 5 insects into a petri dish containing a 9.0-cm Whatman No. 1 filter paper moistened with 700 µL of nematode suspension. For *S. feltiae*, larvae were dissected on the fifth day after infection; for *H. bacteriophora*, larvae were dissected on the seventh and eighth days (before and during matricide). After the larvae were dissected, the mature female nematodes were picked up with an L-shaped handling needle and transferred into a watch glass containing sterile Ringer's solution.

Dilute Hyamine (0.4%) was tested for different durations (1 to 6 min) to surface-sterilize the nematodes. Solution was placed into a sterile watch glass, followed by transfer of nematodes into the watch glass. After sterilization for a set time, nematodes were transferred into sterile distilled water and rinsed for 10–15 s. When sterilization and rinsing were completed, nematodes were placed on NBTA medium and crushed into 2–3 pieces with a sterile loop in order to release their symbiotic bacteria; the resulting macerate was spread across the agar surface. All these procedures were conducted in a sterile flowbox. The petri dishes were placed in an incubator at 28 °C for 48 h to allow bacterial growth. Three replicate plates were prepared for each nematode species.

Single colonies of bacteria showing morphological differences (color, shape, and size) were removed using a sterile loop and transferred to new NBTA agar plates for purification through streak plating. Greenish and greenish-reddish colonies surrounded by clear zones were collected from the pure cultures and examined under light microscope to determine cell morphology.

API 50CH and API 20E strips were used for the biochemical characterization of the isolated bacteria. The catalase test was performed using hydrogen peroxide. For putative *Photorhabdus* species, production of bioluminescence was tested in a dark room.

Bacteria were grown on NBTA for 48 h and then suspended in sterile Ringer's solution (Kaya and Stock, 1997). Five microliters of the bacterial suspension was injected into chilled *G. mellonella* larvae (held at 4 °C for 10 min) with a 1-mL insulin syringe. The same volume of sterile Ringer's solution was used as control. The injected larvae were placed in petri dishes and incubated at room temperature for 4 days (Zhou et al., 2002).

Total bacterial genomic DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN). The 16S rRNA gene was amplified by PCR using primers 16S 20 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S 1390 (5'-GACGGGCGGTGTGTACAA-3'). The final volume of PCR mix was 50 µL, containing 1 µL of DNA, 2 mM MgCl₂, 0.4 pmol of each primer, 0.2 mM dNTP, 1.2 µL of Taq DNA polymerase, and 20 µL 10X buffer. Amplification conditions were 94 °C for 5 min followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C, followed by 15 min at 72

°C. Sequencing was carried out by MACROGEN in Korea. Alignments of the 16S rRNA gene sequences were obtained with CLUSTALW in PAUP software (Swofford, 2003).

Macerates of the nematodes spread onto agar plates after different periods of surface sterilization produced almost-pure cultures of *Xenorhabdus* and *Photorhabdus* bacteria on the agar surface and could be easily purified by fresh streak-plating of characteristic isolates. Bacteria isolated from *Heterorhabditis bacteriophora* produced greenish-reddish colonies and rod-shaped cells, and they showed positive reactions in both catalase and bioluminescence tests. Colonies also had a "sticky" consistency. Those from *Steinernema feltiae* produced green colonies and rod-shaped cells, were catalase-negative, and did not show any bioluminescence activity. When the bacteria were injected into the hemocoel of *Galleria* larvae for the pathogenicity test, all of the larvae died within 24 h, with the cadavers produced from injection of *Heterorhabditis*-associated bacteria turning red, indicative of *Photorhabdus* infection. Twenty typical colonies were examined by microscopy and Gram test. The gram-negative, rod-shaped cells were subjected to further biochemical tests. The API tests indicated that the bacteria isolated from *Steinernema* conformed to *Xenorhabdus bovienii*, and the bacteria from *Heterorhabditis* to *Photorhabdus luminescens* subsp. *thracensis*. The 16S rDNA sequence analyses confirmed the identifications provided by the physical and biochemical tests.

The purpose of this study was to determine a new, simple, and economical method for isolation of symbiotic bacteria from EPNs. We hypothesized that female nematodes, which play a significant role in transferring bacterial symbionts to the next generation at *endotokia matricida*, would be appropriate for isolating the bacterial symbionts. The results demonstrated that dilute Hyamine (0.4%) could be used for fast and reliable sterilization of mature females or hermaphrodite nematodes of the genera *Steinernema* and *Heterorhabditis*, respectively. It was also shown that gravid females/hermaphrodites are a good source for isolation of symbiotic bacteria.

Isolation of the bacteria from IJs requires special equipment like a tissue homogenizer, which can cause contamination if it is not correctly and adequately sterilized. Crushing the IJs can also be challenging due to the resistant structure of the cuticle, whereas female or hermaphrodite nematodes can be easily crushed on NBTA medium by a simple loop.

Extraction of the hemolymph of an infected host (Akhurst, 1980) and the "hanging drop" technique (Poinar and Thomas, 1966) both have a risk of contamination from the intestinal microflora of the insect. While trying to obtain the hemolymph of the insect, its digestive tract can be damaged, and this can easily cause contamination,

whereas there is no such risk while isolating the associated bacteria directly from female nematodes. Ciche et al. (2008) reported that in *Heterorhabditis* spp., during the transmission of the symbiotic bacteria to the IJs, bacteria grow within the nematode's intestinal lumen and are released into the maternal body cavity during *endotokia matricida*, suggesting that *endotokia matricida* is an adaptation for symbiont transmission and thus making it an easy stage for isolation. It is harder to use the *endotokia matricida* stage for *Steinernema* species, because it occurs over a very short period of time and *Steinernema* nematodes in this stage are easily crushed

during the sterilization process. For both *Steinernema* and *Heterorhabditis*, it was also possible to isolate the bacteria from gravid females.

In conclusion, symbiotic bacteria of the entomopathogenic nematodes can be isolated from either gravid females or females at the *endotokia matricida* stage after a short period of surface sterilization, producing symbiont colonies with a low contamination risk.

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