A simple method to collect phoretically active dauer larvae of *Caenorhabditis japonica*

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Caenorhabditis japonica is a bacterial-feeding nematode phoretically associated with the shield bug, *Parastrachia japonensis*. *Caenorhabditis japonica* dauer larvae (DLs) are mainly found on adult female bugs throughout the year, suggesting an intimate association between the nematode and the shield bug, but the mechanisms of the female bug-specific association, as well as the life history of *C. japonica*, are not well known. To understand the biology of *C. japonica* and the behavior of DLs, we developed a simple method to selectively collect phoretically active DLs using swarming behavior. We also demonstrated that the DLs obtained by this method were able to associate with the shield bug. Nematol. Res. 40 (1), 7-12 (2010).

Key words: bacterial-feeding, entomophilic, nictation, phoresis, swarming

INTRODUCTION

The dauer larva (DL) stage of bacterial-feeding nematodes is the stage of facultative diapause. It is the non-feeding third larval stage specialized for dispersal and long-term survival. DLs have altered energy metabolism and arrested development (Burnell *et al.*, 2005; Riddle, 1988). In addition, for phoretic nematodes, DL is an important stage for host searching and association with hosts. Phoretic DLs show some interesting behaviors. Nictation is one of the typical behaviors of phoretic and parasitic DLs for host searching. Nictating nematodes lift their anterior or even more of their body off a moist substrate and wave it in the air (Croll and Matthews, 1977), which seems to improve the chance of phoresy or host encounter (Burr and Robinson, 2004). Swarming, co-ordinated population movement, is observed in many different kinds of nematodes including crowded populations of bacterial-feeding nematodes. During swarming, large numbers of nematodes climb onto each other, forming a writhing structure above the substrate (Burr and Robinson, 2004). The role of the swarming is not well understood but it was suggested to be involved in migration and dispersal (Croll, 1970).

Caenorhabditis japonica Kiontke, Hironaka, and Sudhaus, is a bacteriophagous species closely related to a model organism, *C. elegans* Maupas. While *C. elegans* is a hermaphroditic species and hermaphrodites mate with males in the presence of males, *C. japonica* reproduces only by mating between males and females (Kiontke *et al.*, 2004).

As is the case for other nematodes, there seem to be four larval stages before reaching the adult stage. During *in vitro* culture of *C. japonica*, many DLs observed, as is often seen in other rhabditid nematodes, but conditions affecting the dauer formation in *C. japonica* are still not well understood. Details of the life cycle in *C. japonica* are currently under investigation.

Caenorhabditis japonica has been isolated from a hemipteran insect, the shield bug, *Parastrachia japonensis* Scott (Kiontke *et al.* 2002). *Parastrachia japonensis* is a sub-social insect showing egg-guarding and progressive provisioning behaviors (Tsukamoto and Tojo, 1992). The general life history of *P. japonensis* has been reported (*e.g.* Filippi-Tsukamoto *et al.*, 1995; Gytoku and Tachikawa, 1989; Tachikawa and Schaefer, 1985; Tsukamoto and Tojo, 1992). It is a gregarious insect that spends most of the year in aggregations on a variety of nonhost trees and plants in reproductive diapause. The aggregations are largely dispersed only during the reproductive phase from early May through most of July. After mating in early May, males die off but mated females oviposit a round egg mass in the nest under the litter and keep the mass suspended at the end of the stylets. After hatching, females provision the nest with drupes. Females begin dying off when nymphs become 3rd stadium or later. After passing through five nymphal stadia, new adults begin emerging in late July. New adults do not feed, and they join aggregations of old adults. Since *C. japonica* are mainly found on the body surface under the wings and between the body segments of females of this bug, evolution of this nematode seems to have favored establishing a life history associated with the specific host bug. However, very little information is available on the

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biology of *C. japonica* thus far. To elucidate the intimate association between *C. japonica* and the host bug, studies on the behaviors of *C. japonica* DLs are a key issue.

Behavioral assays are largely influenced by the physiological condition of nematodes. To obtain reproducible results, it is important to establish an active nematode population with uniform physiological conditions. Because DL formation is stimulated by a pheromone and the food supply in *C. elegans* (Riddle, 1988) and probably in other rhabditid nematodes, DLs of rhabditids are usually collected from old plates when nematodes are cultured on an artificial diet under laboratory conditions. However, the DLs obtained from old plates could be a mixture of DLs of different ages and different physiological conditions because DL formation does not always occur at the same time, and the number of DLs gradually increases in culture plates. In addition, not only DLs but also other stages are usually collected from old plates. Non-dauer stages are usually lysed and only DLs can be collected by the SDS-treatment (Riddle, 1988), but such treatment may affect the behavior and physiological condition of DLs by removing components and exudates from the body surface, amphids and phasmids.

Since the host bug of *C. japonica* moves on the litter of a deciduous forest, *C. japonica* DLs apparently have a tendency to use negative geotactic behavior to find a host. In addition, *C. japonica* DLs are very mobile and show possible host searching and phoretic behaviors such as nictation and swarming. By using these characteristics of *C. japonica* DLs, we established a new simple method to selectively collect phoretically active DLs of *C. japonica*.

MATERIALS AND METHODS

Nematodes:

Caenorhabditis japonica strain H1 was established on an NGM plate (Sulston and Hodgkin, 1988) seeded with *Escherichia coli* (Migula) OP50 from nematodes isolated from a female bug collected at Hinokuma Mountain Prefectural Park, a natural secondary forest in Saga, Japan. Eggs were surface-sterilized using alkaline hypochlorite solution (Sulston and Hodgkin, 1988) and hatched larvae were cultured on an NGM plate seeded with *E. coli* OP50.

Culture conditions and collection of DLs:

To obtain large amounts of synchronized DLs, nematodes were cultured on dog food agar (DFA) medium (Hara *et al.*, 1981) seeded with *E. coli* OP50. About 2 g of powdered dog food (VITA-ONE, Nihon Pet Food Co. Ltd., Japan) and 5 ml of 1% water agar was added to a 50 ml glass sample bottle (LABORAN, AS ONE Corp., Japan) and

the bottle was covered with a piece of aluminum foil (Fig. 1A). To collect only DLs, a 200 μ l polypropylene pipette tip (yellow tip; 5 cm long; Watson, Fukaekasei Co., Ltd., Japan) was vertically inserted at the center of DFA medium with the tapered side up. To compare the efficiency of collecting DLs, a 10 μ l pipette tip (white tip, 3 cm long; Axygen Scientific Inc., USA), 1,000 μ l pipette tip (blue tip, 7 cm long; Quality Scientific Plastics Inc., USA), or a 2 ml sampling tube (4 cm long; Axygen Scientific Inc., USA) was used instead of the yellow tip. Bottles containing the medium were autoclaved at 120°C at 1.2 atm for 20 min. After autoclaving and cooling down to room temperature, several drops of *E. coli* OP50 suspension in Luria-Bertani broth were added to the surface of the medium and incubated at 37°C overnight. About 200 nematodes of mixed stages collected from NGM plates were inoculated onto the surface of DFA medium and incubated at 25°C. About 10 days later, nematodes were harvested from DFA medium, treated with 1% SDS for 30 min to kill non-dauer stages and washed well with sterile distilled water at least three times. As a result, non-dauer stages were killed and DLs, which can survive the SDS-treatment, were obtained. About 200 DLs thus collected from the DFA medium were inoculated onto the surface of the medium and incubated at 15°C, 20°C, 25°C, or 30°C under dark conditions. To maintain high humidity, the nematode culture was kept in a 100 ml glass bottle with a lid containing about 10 ml of sterile water (Fig. 1B).

Fig. 1. Culture bottles for nematode propagation and selectively collecting dauer larvae (DLs).

DLs propagated in the dog food agar (DFA) medium crawl up, nictate and swarm at the top of a yellow pipette tip. A: a 50 ml culture bottle containing DFA medium. A yellow pipette tip was put on the surface of DFA medium as shown; B: a 100 ml bottle with a 50 ml culture bottle. To maintain high humidity, sterile water was put in the 100 ml bottle.

Nematode clumps swarming up on the top of each pipette tip were picked up daily using a worm picker (a 1 cm piece of platinum wire mounted on the tip of a Pasteur pipette) and the number of nematodes was counted. Each experiment had five replicates and the trials were repeated three times. Since there were no significant differences among repeats, the data were pooled and used for analyses.

Loading experiment:

Caenorhabditis japonica DLs are found on most female adult bugs in the field. To remove naturally associating DLs and to obtain nematode-free host bugs, adult females of *P. japonensis* collected from Hinokuma Mountain Prefectural Park were partially submerged in tap water for three days before starting loading experiments. Bugs were put in a short stainless steel pipe (7.2 cm dia., 3.4 cm height), one end of which was covered with wire netting (pore size: 1 mm²), and then the pipe with bugs was placed in a plastic box containing tap water (ca. 3 cm depth) with the wire netting side up so that the bugs, grasping onto the wire mesh with their legs, were partially submerged. Thus, with the abdomen facing the wire netting, the dorsal part of the bugs remained touching the water, which allowed the naturally associating *C. japonica* DLs to recover from quiescence, and move into the water. In the preliminary experiments, three-day-partial submergence in tap water was confirmed to be sufficient to remove naturally associating nematodes. After nematode-free bugs were prepared, about 1,000 *C. japonica* DL individuals collected from the top of yellow pipette tips (six to seven days after nematode inoculation) were inoculated onto a 3 cm plastic Petri dish, the bottom of which was lined with filter paper, and then a nematode-free bug was released in the Petri dish. Twenty-four hours later, the bug was dissected and soaked in M9 buffer (Sulston and Hodgkin, 1988) for 3 hr, and the number of nematodes associated with the bug was counted. Ten adult females were used for the loading experiments.

Statistical analysis:

Differences between the mean values of nematode numbers swarming on a pipette tip were analyzed by ANOVA (Kaleida Graph 4.0J, Synergy Software, USA).

RESULTS

Comparison of substrates for swarming:

When DFA medium was used for nematode culture, *C. japonica* propagated well and many DLs climbing up on the inner surface of the glass bottle were observed under uncontrolled humidity conditions at 25°C. To efficiently collect only DLs, we put a substrate for nictation and swarm-

ing at the center of the DFA medium under the same experimental conditions. For choosing the appropriate shape and height for the substrate, we tested white, yellow and blue pipette tips, and a 2 ml sampling tube. When a white tip (3 cm height) was put into the center of DFA medium, a big nematode swarm was observed on the top of the pipette tip but the swarm contained not only DLs but also other stages of nematodes (data not shown). When a yellow tip (5 cm height) was used, a big swarm was observed and large numbers of only DLs were collected (Fig. 2). When a blue tip (7 cm height) was used, only DLs, but smaller numbers of them, were harvested. When a 2 ml tube (4 cm height) was used, very few DLs swarmed and it was difficult to collect DLs (data not shown). Thus, we decided to use a yellow pipette tip to collect DLs from DFA medium.

Effects of temperature and humidity on swarming of DLs:

Caenorhabditis japonica propagated well at 20°C and 25°C. Swarming DLs were seen from the top of the yellow tip from four to five days after nematode inoculation. The number of DLs recovered from the top of the yellow tip reached a maximum at 12 days and six to seven days after nematode inoculation at 20°C and 25°C, respectively (Fig. 3). *Caenorhabditis japonica* did not propagate well at 15°C or 30°C (data not shown). When *C. japonica* was cultured under high humidity, the number of DLs collected from the top of the yellow tip was larger than that without controlling the humidity (Fig. 3). About 2,500 DLs could be harvested from the top of a yellow tip 7 days after nematode inoculation.

Loading experiment:

Twenty-four hours after DLs were exposed to bugs, the bugs were dissected and DLs were mainly found under the wings and between the body segments of all the bugs where naturally associated DLs were usually found. Mean nematode number recovered from a female bug was 97 ± 83 (17-314).

DISCUSSION

In this paper, we established a simple method to collect phoretically active *C. japonica* DLs of similar ages, and demonstrated that the DLs collected by this method associated with the host bug. For induction of swarming and collection of DLs, a yellow pipette tip (5 cm height) was the best among the substrates tested. A shorter tip (3 cm height) was not high enough to eliminate other larvae and adults and a longer tip (7 cm) was too high for the DLs to swarm up so not many were collected. When a 2 ml sampling tube (4 cm height) was tested, very few swarming

Fig. 2. Comparison of numbers of dauer larvae (DLs) obtained from the top of pipette tips. A clump of DLs swarming on the top of a pipette tip was picked up at 24-h intervals and the number of nematodes in the swarm was counted. Each bar indicates standard deviation ($n = 15$).

Fig. 3. Effects of temperature and humidity on the swarming of dauer larvae on the top of a yellow pipette tip. Each bar indicates standard deviation ($n = 15$). * Significant difference between the condition (ANOVA, $P < 0.01$).

nematodes were observed. Since the top of the 2 ml tube was not as sharp as a pipette tip, DLs might have moved down, rather than stay on the top of the 2 ml tube. These results indicate that not only the height but also the shape of the top of the substrate seem to be important factors for nictation and swarming of DLs. In this paper we chose polypropylene pipette tips and tubes for experiments simply because they are inexpensive, uniform, easy to obtain, and easy to set on the DFA medium. As a result, we found

that the yellow pipette tip tapered to a point was a good substrate, having an appropriate size and shape for induction of swarming and collecting DLs.

The number of DLs collected from the top of a pipette tip was influenced by temperature. This is probably due to the propagation and dauer formation of *C. japonica* at the different temperatures. Temperatures below 15°C or above 30°C were apparently inappropriate for the growth of *C. japonica*. The total number of DLs collected was greater at

20°C than 25°C under uncontrolled humidity conditions, but larger numbers of DLs were collected earlier at 25°C. Nematodes propagate faster at 25°C and DLs can be induced earlier at 25°C than at 20°C since dauer induction is influenced by temperature, nematode density and food supply (Riddle, 1988). Humidity also appears to be an important factor for induction of swarming. During the course of these experiments, we observed larger numbers of DLs swarming on the top of pipette tips on relatively humid medium than on dried medium. Thus, we put the DFA medium in a bottle with water to keep the humidity high. As a result, we found that the high humidity enhanced the number of swarming DLs (Fig. 3).

Swarming behavior occurs in some bacteriophagous, mycophagous and plant parasitic nematodes (Croll, 1970; McBride and Hollis, 1966), but the role and the mechanisms of swarming are not well understood. Wharton (2004) suggested that clumps formed following swarming may aid in desiccation survival of these nematodes but swarming itself is not triggered by desiccation. Womersley *et al.* (1998) suggested that swarming in these nematodes is probably triggered by the accumulation of toxic waste products, the exhaustion of food supplies and/or the senescence of a host plant. In the case of *C. japonica* DLs, induction of DLs appears to be the result of an accumulation of dauer inducing chemicals and exhaustion of food supplies, but swarming itself does not appear to be directly triggered by these factors. Since we observed that the swarming of DLs was initiated by nictation and aggregation of nictating DLs, the swarming in *C. japonica* DLs may be an important behavior for host searching, initial association with the host and/or survival on the host. In *C. elegans*, aggregation behavior of feeding stages has been analyzed at molecular and neural levels (*e.g.* de Bono and Bargmann, 1998; Persson *et al.*, 2009; Rogers *et al.*, 2006). *Caenorhabditis elegans* wild strains switch between two foraging behaviors, solitary or social feeding (aggregation) behavior, in response to subtle changes in ambient oxygen. This switch is conferred by a naturally variable neural globin GLB-5, and is modified by the naturally variable neuropeptide receptor NPR-1. In our study, we observed swarming behavior of *C. japonica*. Usually DL is the non-feeding and dispersal stage and *C. japonica* DLs were observed to disperse on the culture bottle, eventually climbing up the bottle's wall. This dispersal behavior of *C. japonica* DLs might be controlled by using a mechanism similar to that seen in *C. elegans*, which involves sensing the oxygen concentration. However, the swarming we observed in *C. japonica* appears to be quite different from the aggregation behavior observed in *C. elegans* because it is a result of an accumula-

tion of nictating DLs that dispersed but climbed up on the yellow tip. Further studies are necessary for understanding the swarming behavior in *C. japonica* DL.

Although the biology and life history of *C. japonica* are poorly understood thus far, we have observed some interesting phenomena of *C. japonica*: female bug-specific association and seasonal changes of *C. japonica* DLs on female bugs (Tanaka *et al.*, unpublished data). *Caenorhabditis japonica* DLs are usually found on most female bugs of *P. japonensis* as a quiescent form but not from female bugs showing provisioning behavior, which indicates that DLs leave bugs, possibly for propagation, during the reproductive period of the shield bug. Since association with and dissociation from the shield bug are opposite behaviors for DLs, DLs have to change their behaviors depending upon the environmental conditions as well as their physiological condition. There is no information available on the control of such complex behaviors. Careful investigation of such changes in behavior using conditioned DLs is crucial. In addition, DLs found on the bugs are usually in quiescence but details of the physiological quiescent state in nematodes are poorly understood. Using the DLs collected by the method we established, we will be able to explore in greater detail those behaviors and mechanisms of behavioral and physiological changes. Furthermore, the method we developed is not limited to use with *C. japonica* DLs. It can be modified and applied to other nematodes showing active upward movement to prepare nematodes having similar conditions for behavioral and physiological studies.

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