Video Article

Visualizing Bacteria in Nematodes using Fluorescent Microscopy

Kristen E. Murfin[1], John Chaston[1], Heidi Goodrich-Blair[1]

[1]Department of Bacteriology, University of Wisconsin-Madison

Correspondence to: Heidi Goodrich-Blair at hgblair@bact.wisc.edu

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Abstract

Symbioses, the living together of two or more organisms, are widespread throughout all kingdoms of life. As two of the most ubiquitous organisms on earth, nematodes and bacteria form a wide array of symbiotic associations that range from beneficial to pathogenic [1-3]. One such association is the mutually beneficial relationship between *Xenorhabdus* bacteria and *Steinernema* nematodes, which has emerged as a model system of symbiosis [4]. *Steinernema* nematodes are entomopathogenic, using their bacterial symbiont to kill insects [5]. For transmission between insect hosts, the bacteria colonize the intestine of the nematode's infective juvenile stage [6-8]. Recently, several other nematode species have been shown to utilize bacteria to kill insects [9-13], and investigations have begun examining the interactions between the nematodes and bacteria in these systems [9].

We describe a method for visualization of a bacterial symbiont within or on a nematode host, taking advantage of the optical transparency of nematodes when viewed by microscopy. The bacteria are engineered to express a fluorescent protein, allowing their visualization by fluorescence microscopy. Many plasmids are available that carry genes encoding proteins that fluoresce at different wavelengths (*i.e.* green or red), and conjugation of plasmids from a donor *Escherichia coli* strain into a recipient bacterial symbiont is successful for a broad range of bacteria. The methods described were developed to investigate the association between *Steinernema carpocapsae* and *Xenorhabdus nematophila* [14]. Similar methods have been used to investigate other nematode-bacterium associations [9,15-18]and the approach therefore is generally applicable.

The method allows characterization of bacterial presence and localization within nematodes at different stages of development, providing insights into the nature of the association and the process of colonization [14,16,19]. Microscopic analysis reveals both colonization frequency within a population and localization of bacteria to host tissues [14,16,19-21]. This is an advantage over other methods of monitoring bacteria within nematode populations, such as sonication [22]or grinding [23], which can provide average levels of colonization, but may not, for example, discriminate populations with a high frequency of low symbiont loads from populations with a low frequency of high symbiont loads. Discriminating the frequency and load of colonizing bacteria can be especially important when screening or characterizing bacterial mutants for colonization phenotypes [21,24]. Indeed, fluorescence microscopy has been used in high throughput screening of bacterial mutants for defects in colonization [17,18], and is less laborious than other methods, including sonication [22,25-27]and individual nematode dissection [28,29].

Video Link

The video component of this article can be found at https://www.jove.com/video/4298/

Protocol

1. Construction of a Fluorescent Bacterial Strain via Conjugation

1. Grow the recipient strain (symbiont to be examined) and donor strain overnight. The donor strain, usually *Escherichia coli*, should be capable of donating DNA through conjugation and should be transformed with a plasmid (**Table 2**) that carries a gene encoding a fluorescent protein. Depending on the plasmid, a conjugation helper strain may also be required. If so, this strain should also be grown overnight. The donor strain and helper strain should be grown with antibiotics to select for maintenance of the plasmid.
2. Subculture the donor, helper, and recipient strains into nutrient rich growth media lacking antibiotics in a 1:100 ratio of culture to medium.
3. Grow the cultures at the temperature appropriate for each strain until they reach mid-log stage growth (OD_{600}~0.6). For *Xenorhabdus* and *E. coli* this stage of growth is reached approximately 3 to 4 hr after subculturing. This may vary depending on the strain, or in some cases the plasmid, used.
4. Mix the strains in a microcentrifuge tube and centrifuge for 2 min at 17,900 x g (13,000 rpm in most microfuges). The proportion of donor and recipient that yields best results will vary for different combinations, and may have to be empirically determined. For a *Xenorhabdus* recipient and *E. coli* donor, we use a 3:1 or 1:1 ratio. If a helper strain is needed (*e.g. E. coli*), it should be added in the same ratio as the donor.
5. Decant the supernatant.

6. Re-suspend the cell pellet in 30 µl of fresh media.
7. Spot the suspension onto a nutrient rich media plate without any antibiotics, and allow the spot to dry.
8. Incubate the plate, inverted, overnight at a temperature optimal for the recipient bacterium and permissible for the donor (and helper, if applicable). The plate should be incubated at least 18 hr.
9. Scrape up the spot and streak for single colonies on a selective antibiotic plate. The antibiotics should select against the donor and for the recipient containing the plasmid. One or two additional isolation streaks may be necessary to isolate a pure culture.
10. Ensure the resulting colonies are the recipient symbiont, and not the donor strain, by screening for the presence of recipient specific phenotypes, such as colony morphology, or polymerase chain reaction detection of recipient-specific genes. Screen the colonies for presence of the plasmid by polymerase chain reaction of a known plasmid sequence and fluorescence. The colonies should fluoresce under the correct wavelength corresponding to the fluorescent protein.

2. Production of Axenic Nematode Eggs

1. Grow 5 ml of the natural bacterial symbiont overnight. To start a culture, bacteria may be inoculated into Lysogeny Broth (LB) or other culture media directly from a freezer stock or from a streak plate.
2. Spread 600 µl of the bacterial culture onto 10 mm Lipid Agar (LA) plates [29]. Eight to ten plates will yield enough nematodes for most species.
3. Incubate in the dark without moisture at 25 °C for two days.
4. Add 5,000 infective juvenile nematodes, or other stages depending on the nematode being used, in 500 µl of media to the bacterial lawns. Incubate the plates at 25 °C for 3 days.
5. Check your plates for the presence of adult nematodes and eggs. Place 20 µl of water onto a microscope slide. Using a sterile stick scrape-up a small amount of nematodes from the bacterial lawn. Place the stick into the water and allow the nematodes to swim off. Look at your slide under low magnification. For more information on appearance see the discussion and **Figure 2**.
6. If eggs and females are visible, continue; otherwise, incubate the plates at 25 °C and check for proper development every 6-12 hr. When females contain eggs, place a few ml of water on the surface of the plates. Gently swirl the plates and pour the water into a 50 ml conical tube. Nematodes should come off of the plates and no longer be visible on the plate surface. Several rinses may be necessary.
7. Allow the nematodes to settle to the bottom of the tube.
8. Rinse nematodes. Pipette off the excess water from the top of the tube. Refill with clean water and allow adult nematodes to settle. Pipette off the excess water.
9. Fill the conical tubes with egg solution. Once egg solution is added the timing of all steps with egg solution must proceed exactly as described for maximum egg yield. Incubate the tubes while gently shaking for exactly 10 min. Most nematodes should be dissolved by the end of the incubation.
10. Immediately centrifuge the conical tubes at 1250 x g for exactly 10 min (this includes bringing the centrifuge up to speed but not down to 0 x g. Use the brake).
11. Immediately decant the supernatant.
12. Immediately re-suspend the pellet with egg solution by pipetting.
13. Immediately fill conical tube with egg solution and mix well by inverting 3-5 times. Then immediately spin as in 2.10.
14. Immediately decant the supernatant.
15. Re-suspend the pellet in LB by pipetting, transfer to a 15 ml conical tube for improved pelleting during wash steps. LB is standard for *Steinernema* spp. Other buffers (*e.g.* a minimal salts medium) may be used depending on the nematode and bacterium, keeping in mind the buffer must not harm either the nematode or bacterium.
16. Fill the tube with LB and spin as in 2.10.
17. Decant the supernatant, and re-suspend in LB.
18. Rinse the nematodes a total of 3 times by repeating 2.14 and 2.15.
19. Dilute the re-suspended nematode eggs to an appropriate volume. There should be at least 10 eggs per microliter. Eggs can be stored in a 6-cm Petri dish in 5 ml LB for at least four days by adding antibiotics that inhibit growth of colonizing bacteria and wrapping the plate in parafilm. The eggs will need to be washed once in 15 ml LB (as in 2.13 and 2.14) prior to inoculating onto bacterial lawns. Note that eggs will hatch during this time and may not be synchronized developmentally when used in colonization assays.

3. Co-cultivation Assay with Fluorescent Bacteria

1. Grow fluorescent bacteria overnight, selecting for the plasmid if necessary.
2. Spread 600 µl of the bacterial culture onto 10 mm Lipid Agar plates with a sterile stick. Incubate at 25 °C for 2 days.
3. Place 500-5,000 axenic nematode eggs in a total of 100 to 400 µl (optimally 2,000 nematodes in 200 µl) onto each lipid agar plate.
4. Incubate in the dark without moisture at 25 °C until IJs, or other stages of interest, form. IJs will be visible as a fuzzy white ring on the edge of the plate. To look at other life stages, see Protocol 4.
5. Place the plates in a modified White trap[30]. Remove the lid of the lipid agar plate and place the bottom into the bottom of an empty 100 mm x 20 mm Petri dish. Fill the large Petri dish with water, or other buffer appropriate to your nematode, to surround the small plate. The water level should be approximately half the height of the small plate.
6. Incubate the water trap until progeny IJs have emerged into the buffer.
7. Infective juvenile nematodes can be stored in water in a tissue culture flask.

4. Collection of Early Life Stages for Screening

1. Use steps 3.1 through 3.4 to set up the assay.
2. Incubate the plates until the desired life stages are present. Daily visual inspection may be necessary to determine timing. To inspect the plates, use the procedure described in Protocol 2.5. For *S. carpocapsae* eggsgrown with *X. nematophila* on LA plates, gravid females will be present in 4-5 days, juveniles will be present in 7 days, and infective juveniles will start to be produced by 15-17 days.

3. When the desired life stages are present, collect your sample. Fill a well of a 96 well plate with 200 µl of PBS or other appropriate buffer. Gently scrape off some of the bacterial lawn that contains nematodes. A pea-sized amount (~50 mg) will provide at least several hundred nematodes once F1 progeny are produced, but more may be necessary for earlier time points. Place the stick into the well containing buffer.
4. Incubate for 30 sec to a minute. Remove the stick and scrape off the remaining residue. Use the stick to remove any agar from the well. The nematodes should be visible within the buffer. Move the buffer mixture to a clean microfuge tube.
5. Treat the nematodes with levamisole or other paralyzing agent. Dissolve a few grains of levamisole into 30 µl of water. Add 1-2 µl of this mix for each 50 µl of sample. After levamisole treatment the nematodes will be non-viable.
6. If samples will be viewed at a later date, fix as described in this step. If not, skip to step 4.6. Add 200 µl of fixative solution containing 1X buffer and 4% paraformaldehyde. Mix gently, pipetting may cause delicate life stages to lyse. Cover with foil, and incubate at room temperature on a shaker on low for at least 18 hr.
7. Place the tubes in a tube rack, and allow the nematodes to settle to the bottom of the tube.
8. Rinse nematodes to remove background. Pipette off excess liquid and add 200 - 500 µl of buffer and gently mix. Repeat. This may be repeated a few times to remove more background if desired.
9. Allow the nematodes to settle to the bottom of the tube and re-suspend in the desired volume.
10. If the nematodes are not fixed, screen immediately. Fixed nematodes may be stored in the refrigerator for up to two weeks. Store in the dark.

5. Screening Nematodes for Bacterial Association by Microscopy

1. Select some nematodes to view under the microscope by removing a small sample of your mixture.
2. To ensure that the nematodes are still for the picture, treat with a paralytic agent as described in Protocol 4.5. If you are not taking a picture or the nematodes are already fixed, this step may be skipped.
3. Add 20-30 µl of nematodes in water to your microscope slide and place a coverslip on top.
4. View whole nematodes using light microscopy to ensure nematodes are in the field of view.
5. View nematodes using the fluorescent setting on the microscope that corresponds to the fluorescence expressed by the bacteria.
6. To identify bacterial localization, take photos of the nematode under fluorescent setting and a light microscopy setting, and then superimpose the images. The photos need to be of the same field of view. It may be necessary to try several magnifications and views of the nematode to find the bacteria.
7. The distribution of nematode colonization can be quantified by counting a population of nematodes, scoring for the presence or absence of bacteria, and calculating the percent colonized. We recommend counting until at least 30 nematodes within the population are counted in each category (with or without colonization) to obtain a reliable value.
8. When only a few nematodes in the population are colonized, it may be necessary to count several thousand nematodes before 30 are observed. For high-throughput counting use the following steps.
9. Instead of counting nematodes individually, aliquot the population into a multi-well plate (*e.g.* a 24-well plate). After the nematodes have settled to the bottom of the dish, each well can be scanned on the microscope and the number of colonized nematodes can be scored.
10. The total number of nematodes in a population can be identified by serial dilutions of nematodes in the well. For example, 1 ml of nematodes can be added to a well, mixed well by stirring with a pipet tip, and 3 µl can be removed and counted for quantification of the total population in the well.
11. The percentage of colonized nematodes can be obtained by dividing the number colonized by the total number of nematodes in the well.

6. Representative Results

Example microscope images of *Steinernema* nematodes associated with *Xenorhabdus* bacteria are shown in **Figure 3**. To create the composite image seen in **Figure 3A**, a phase contrast image was overlaid with a fluorescent image. The arrow in **Figure 3A** indicates the bacteria present within the infective juvenile nematode (bar = 100 µm). **Figure 3B** was constructed in a similar manner and depicts a juvenile nematode with green fluorescent protein labeled bacteria (green rods) localized throughout the nematode intestinal lumen (bar = 20 µm). A population of the nematodes from two media were counted and scored for colonization by the bacterial symbiont (**Table 1**). For robust statistics, it is best to count at least 100 nematodes per sample with at least 30 falling in each category. As seen in **Table 1**, these nematodes are colonized at a level of approximately 14.6% when grown on lipid agar and 68.6% when grown on liver kidney agar. Other nematode and bacterial species have been shown to have different levels of colonization. For example *X. nematophila* colonizes 99% of *S. carpocapsae* infective juveniles (Martens 2003), and *P. luminescens* colonizes 26% of *H. bacteriophora* infective juveniles (Ciche 2003).

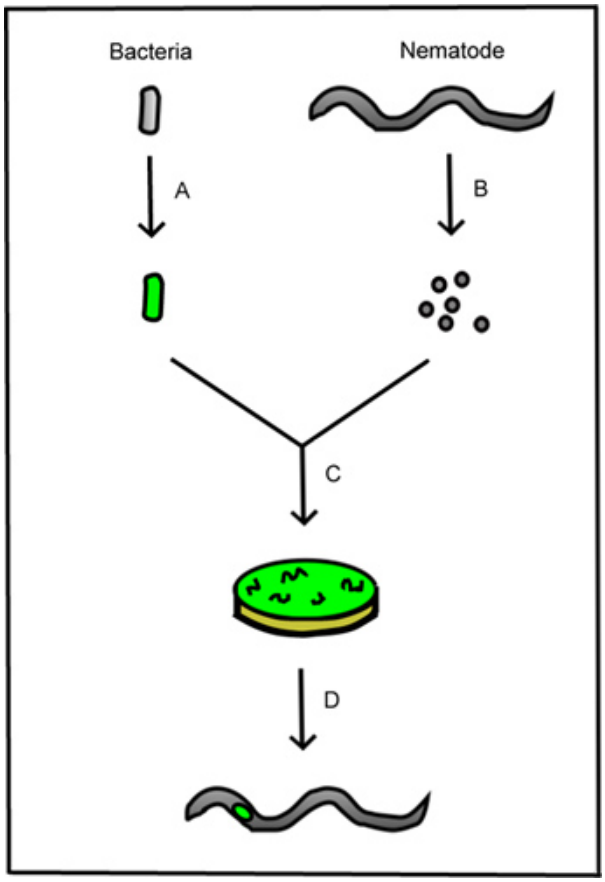

Figure 1. Schematic outline of the method. A. The bacterium is engineered to express a fluorescent protein. **B.** Nematode eggs are isolated from adult nematodes to produce sterile nematodes. **C.** The sterile nematodes are co-cultivated with the fluorescent bacteria. **D.** The resulting life stages are viewed under a microscope to evaluate bacterial presence within the nematode.

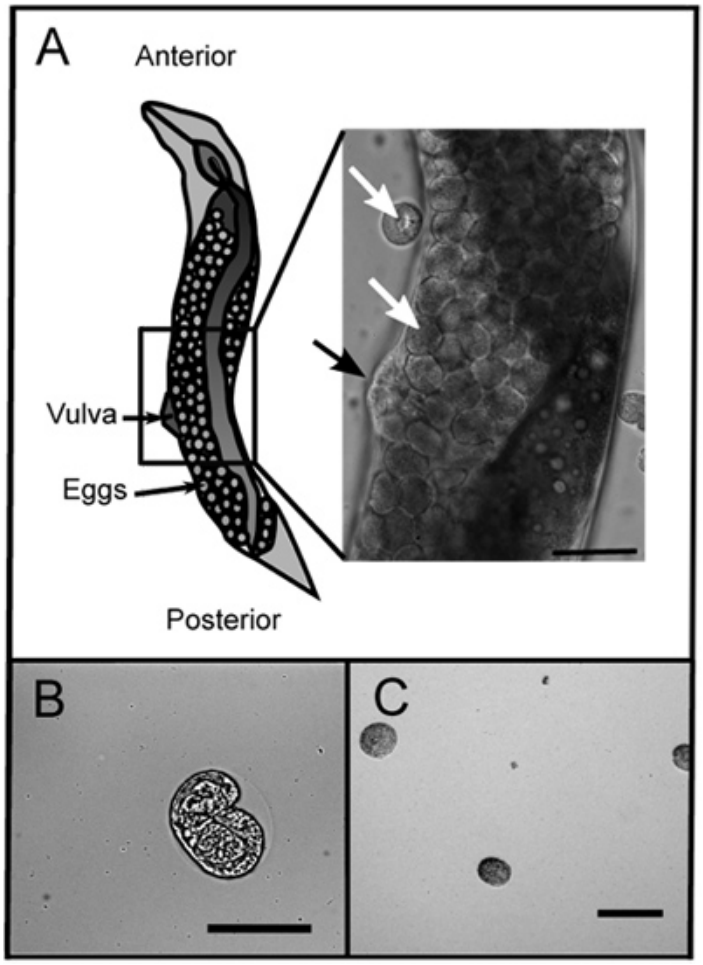

Figure 2. Depiction of adult females containing eggs. A. The schematic shows the general appearance of *Steinernema* females. Inset: DIC image of a *S. feltiae* gravid female. The black arrow indicates the vulva. White arrows show visible eggs. Image is at 20X magnification, and the scale bar represents 100 µm. **B.** DIC image of a developed but unhatched *S. feltiae* nematode egg. Image is 40X magnification, and the scale bar represents 50 µm. **C.** DIC image of eggs isolated from *S. feltiae* nematodes under 10X magnification. Scale bar is 100 µm.

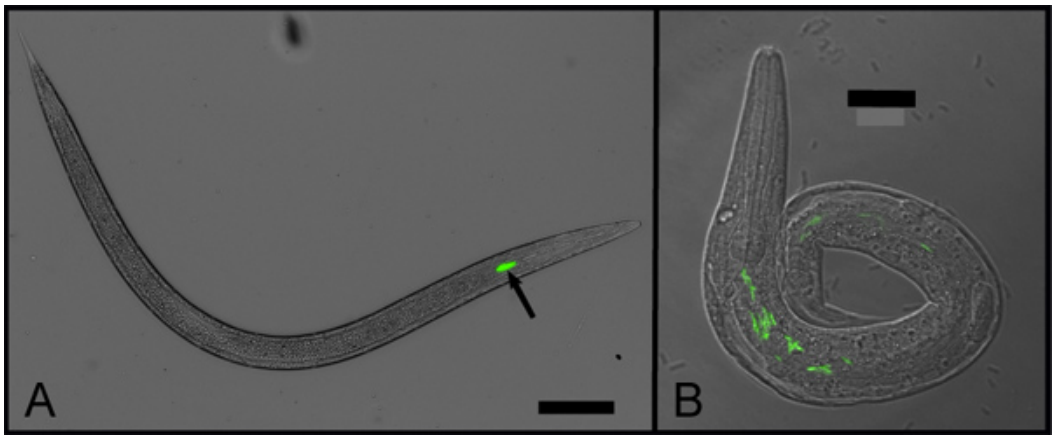

Figure 3.Example microscope images of nematode-bacterial association. A. *S. puntauvense* nematodes were associated with their bacterial symbiont, *X. bovienii*, expressing GFP. The image is a composite image produced by overlaying a phase contrast image with a fluorescent image from the same field of view. The arrow indicates the fluorescent bacterial symbiont within the nematode host. Scale bar represents 100 µm. **B.** This image shows a *S. carpocapsae* juvenile nematode with GFP-expressing *X. nematophila* localized within the nematode intestine. This image was constructed through overlaying a fluorescent image over a differential interference contrast image. The scale bar represents 20 µm.

Strain	Number of Nematodes With acteria	Total Nematodes Counted	Percent of Nematodes olonized

S. puntauvense Lipid Agar	30	205	14.60%
S. puntauvense Liver Kidney Agar	72	105	68.60%

Table 1. Example scoring of a nematode population for bacterial presence. In this experiment, axenic *S. puntauvense* nematodes were grown with their GFP-expressing symbiont on different growth media (lipid agar and liver kidney agar [32]) to test for colonization defects. A total of at least one hundred nematodes per sample were counted and scored for the presence of bacteria. For statistical power, three experimental replicates should be counted with at least 30 nematodes falling in each category.

Plasmid Name	Fluorophore	Antibiotic Cassette	Insertion Site	Special Instructions	Source
mini-Tn7-KSGFP	green fluorescent protein	Kanamycin	Tn7 site, upstream of *glmS*	Requires helper plasmid	33
pECM20	green fluorescent protein	Chloramphenicol	Chromosomal insertion	Specific to *X. nematophila* (nucleotide 825459-824847)	13
pBK-miniTn7-ΩGm-DsRed	Ds red fluorescent protein	Gentamycin	Tn7 site, upstream of *glmS*	Requires helper plasmid	31
pTurboRFP-B	TurboRFP	Ampicillin	Extrachromosomal, ColE1 replicon	Origin may not replicate in all bacteria	Evrogen
pPROBE-GT	green fluorescent protein	Gentamycin	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32
pPROBE-KT	green fluorescent protein	Kanamycin	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32
pPROBE-NT	green fluorescent protein	Kanamycin	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32
pPROBE-AT	green fluorescent protein	Ampicillin	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32
pPROBE-TT	green fluorescent protein	Tetricycline	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32
pPROBE-OT	green fluorescent protein	Streptomycin	Extrachromosomal, pVS1/p15a replicons	Origin may not replicate in all bacteria	32

Table 2. Fluorescent protein containing plasmids. A list of potential plasmids for insertion of a fluorescent protein into the bacterial symbiont is given listed by name of plasmid. Other information included are the fluorescent protein encoded, antibiotic cassette used for plasmid maintenance, other instructions for use, the source of the plasmid. The concentration noted in parentheses is the concentration of the antibiotic used for *X. nematophila*. Each of these plasmids has been used successfully in either *Xenorhabdus* or *Photorhabdus*. Additional information can be obtained from the noted citations. Depending on the bacterium being tested, some plasmids may not work based upon the fluorescent protein, antibiotic selection, insertion site, or origin of replication. The plasmids listed above contain different features that may enable use in the bacterium of interest. For example, mini-Tn7-KSGFP inserts into the attTn7 site of the chromosome, while pECM20 inserts into the *X. nematophila* chromosome by homologous recombination. Alternatively, the pPROBE plasmids are maintained extrachromosomally, and each pPROBE plasmid has the same backbone and fluorophore but have different selectable markers or origins of replication to enable their use in a variety of taxonomic or mutant backgrounds.

Discussion

The protocol described here provides a method for the optical detection of bacteria within a nematode host (**Figure 1**). This method takes advantage of the optical transparency of nematodes and the ability to fluorescently label bacteria, enabling *in vivo* analysis of bacteria within the nematode host (**Figure 3**). Specifically, this approach identifies bacterial localization within its host. By counting a nematode population and scoring for bacterial presence, the frequency of bacterial colonization across the nematode population can be determined (**Table 1**). This method is one of many potential techniques that can be utilized for studying the interactions between nematode hosts and bacterial symbionts. Related methods have been described previously to isolate the bacterial symbiont, grow nematodes axenically, and manipulate both partners [25-27].

The protocol described here was developed in the *Xenorhabdus nematophila-Steinernema carpocapsae* model system [14], and similar approaches have been used in other entomopathogenic nematode-bacterial associations [9,15,17,18]. Several conditions must be met in order to apply this method to other nematode-bacterial systems. First, the bacterial symbiont must be able to be isolated from the host and grown independently in culture. Second, the symbiont must be able to take up DNA through transformation or conjugation in order to introduce the fluorescent protein. Third, for the best results, the nematode must have a stage that can be made axenic and reintroduced to bacteria. However, even if the nematode cannot be isolated from the bacteria it might still be possible to visualize the association: instead of adding axenic eggs to the lawn of fluorescent bacteria, add a conventionally raised life stage and proceed with the protocol as described. Any results will suffer from the caveat that bacteria that do not express GFP will compete with GFP-expressing bacteria for localization to specific nematode tissues, and because of this, colonization should not be measured by microscopic counting. However, unless the bacteria that do not express GFP drastically outcompete GFP-expressing bacteria, GFP-expressing bacteria should localize to nematode tissues in at least some animals in the population and suggest important tissue sites for bacterial colonization. A long-standing caveat of using fluorescent-protein expressing strains is that they may colonize a host with different efficiency than a strain that does not express fluorescent proteins (*e.g.* [12]).

Steps described in this protocol may require optimization depending on the nematode and bacterial species that are being used. For conjugation of the bacterium some conditions that can be altered are the length and temperature of growth, the ratio of donor to recipient, and plasmid used. Examples of relevant plasmids are listed in **Table 2**. All of these plasmids have been successfully used in either *Xenorhabdus* or *Photorhabdus* bacteria in association with their nematode host [14,17,20,24,33-35]. To ensure stable maintenance of the fluorescent protein during nematode colonization it is best to use a plasmid that will insert into the chromosome of the target bacterium. Further, some bacteria may not take up these plasmids. For example, pECM20 has only been used in *X. nemtaophila* and very closely related bacterial strains [14] because this plasmid

inserts into the chromosome using a homologous region between the plasmid and chromosome; the plasmid will not insert if the target bacterium lacks this region. To use this plasmid for other bacteria, the *X. nematophila*-specific region must be substituted with a genomic region from the target bacterium. Some plasmid-transformation schemes will also require certain alterations from Protocol 1. For example, mini-Tn*7*-KSGFP and pBK-miniTn*7*-ΩGm-DsRed require a helper plasmid containing transposition genes (*tsnABCDE*)[33,35,36] to insert into the bacterial chromosome. Conjugation is most efficient with these Tn*7*-based plasmids, when 2-hour cultures (~OD_{600}0.3-0.4) are mixed in equal ratios.

After successful conjugation it is important to utilize correct selective plates corresponding to the plasmid used. The selective plates should contain the antibiotic encoded on the plasmid to select for the presence of the plasmid in the recipient and a counter-selection against the donor and helper strains. For example, when conjugating pECM20 in *X. nematophila* the counter-selection used is ampicillin because *X. nematophila* is ampicillin resistant and the donor strain is ampicillin sensitive. If antibiotic counter-selection is unavailable in your system, a diaminopimelic acid (DAP) requiring donor may be used. To grow DAP-requiring strains, DAP is added to solid and liquid growth media during pre-conjugation and conjugation steps, and omitted during counter-selection stages. When DAP is absent the donor strain will not grow and is effectively counter-selected.

To ensure successful egg isolation it is essential to accurately check for egg production (**Figure 2**). At the time of isolation, female nematodes should be full of eggs and some eggs should be visible in the media (**Figure 2A**). If female nematodes are producing fertilized eggs at least some supernatant eggs will have visibly developed as unhatched nematodes (**Figure 2B**). For *S. carpocapsae*, the eggs will change from spherical to slightly oblong prior to developing nematodes and hatching. The timing or conditions of nematode growth may also need to be altered to maximize fertilized egg yield, including shortening or lengthening the period of time before egg harvesting, or using alternate media appropriate to the specific nematode species. At the end of the isolation, eggs should be easily visible within the buffer (**Figure 2C**). Parameters in the egg isolation protocol that may require optimization include bleach and KOH concentrations in the egg solution, incubation time in egg solution, or centrifugation speed. If the egg isolation produces eggs but no nematodes develop during the co-cultivation, it is possible that the isolated eggs are non-viable. To check for viability, leave the isolated eggs in buffer and wait for nematodes to hatch. The eggs should hatch within one or two days of isolation and then the juvenile nematodes can be used in the co-cultivation assay. If the eggs hatch but do not develop during the co-cultivation assay, it may be necessary to change the growth conditions or medium used for co-cultivation. We note that previous studies have isolated bacteria-free nematodes by soaking the nematodes in an antibiotic cocktail (*e.g.* [3,37]). The approach we describe here is free of some of the caveats of antibiotic soaking, including complications with using bacteriostatic antibiotics, antibiotic-resistant contaminating bacteria, and antibiotic effects on the nematodes. Certain stages of nematodes may also be resistant to antibiotics and retain their bacterial symbionts[3]. Finally, we note that for microscopy, the concentration of levamisole necessary for nematode immobilization may vary with different nematode clades.

Once this method has been established in the system of interest, it is possible to alter the technique to derive more information. By looking at earlier time points in the nematode life cycle (Protocol 4), one may be able to identify how the bacterium and nematode initiate their association [14,16]. In addition, this approach can be used to conduct a high throughput mutant screens to identify bacterial factors involved in the symbiosis, using fluorescence to detect colonization [17,18]. Other methods such as signature tagged mutagenesis require the use of radioactively labeled probes and are more time consuming [22,28]. Therefore, the use of fluorescence microscopy for bacterial symbiont visualization in nematodes is an effective and efficient screening tool for investigating bacterial interactions with a nematode host.

Disclosures

No conflicts of interest declared.

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