HHS Public Access

Author manuscript

Curr Protoc Toxicol. Author manuscript; available in PMC 2021 June 01.

Published in final edited form as:

Curr Protoc Toxicol. 2020 June ; 84(1): e94. doi:10.1002/cptx.94.

Generating bacterial foods in toxicology studies with *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans (*C. elegans*) are free living animals that are used as a powerful experimental model in biological sciences. The natural habitat of the animal is the area rich in material from rotting plants or fruits being decomposed by a growing number of microorganisms. The ecology of *C. elegans*' natural habitat is a complex interactive network involving many species, including numerous types of bacteria, virus, fungi, slugs, snails and isopods, among which, bacteria play multifaceted roles in the natural history of *C. elegans*. Under laboratory conditions, *C. elegans* are routinely cultured in a petri dish filled with solidified agar and seeded with the *Escherichia coli* (*E. coli*) strain OP50, the latter offering an alternative model to study the interaction between bacteria and host. Because of the clear advantages of generating specific bacterial foods for mechanistic studies in *C. elegans*, it is important to develop a robust protocol to generate high-quality bacterial foods commensurate with experimental requirements. Based on previous work by us and others, herein, we present a protocol on how to generate these optimal bacteria-food based research tools.

Keywords

C. elegans; *E. coli*; bacteria; liquid culture

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Introduction

The natural habitats of the model organism *C. elegans* are often found in the temperate humid areas that are rich in material from rotting plants (stems and vegetables) or fruits being decomposed by a growing number of microorganisms (Schulenburg & Felix, 2017). It is conceivable that the environment supplies the animal with at least two essential materials: water and an abundance of bacterial food. However, *C. elegans* encounters a much more complicated environment in nature, which exhibits a strong selective constraint for the animal (Frezal & Felix, 2015). Emerging discoveries have shown that the ecology of *C. elegans*' natural habitat is a complex interactive network involving many species, including numerous types of bacteria, virus, fungi, slugs, snails and isopods, among which, bacteria play multifaceted roles in the natural habitat of *C. elegans* (Kiontke & Sudhaus, 2006; Petersen et al., 2015; Schulenburg & Felix, 2017). They provide nutrition for growth and maintenance of health, components of microbiome in the intestine and cuticle that are important for innate immunity, and pathogens that can be harmful to the animal (Schulenburg & Felix, 2017).

In the natural environment, the microbiome in *C. elegans'* intestine and surface of cuticle are important for reproductive growth and immunity (Frezal & Felix, 2015). Analogous to the beneficial effects of microbiome in the intestine of mammals, bacteria from the microbiome in *C. elegans* are believed to have a coevolution relationship with the species, and the interaction between *C. elegans* and its microbiome probably invoke most of the *C. elegans*' genes with currently unknown functions (Frezal & Felix, 2015). In spite of this, some bacteria have been well characterized for their biological roles in the *C. elegans* response to stress, life-span, population growth, and fecundity (Samuel, Rowedder, Braendle, Felix, & Ruvkun, 2016). It is of note that some detrimental or pathogenic bacteria were utilized to establish an alternative model to study innate immunity in *C. elegans* (Hodgkin, Felix, Clark, Stroud, & Gravato-Nobre, 2013), in which the beneficial bacteria from the microbiome were also demonstrated to play a defensive role against the pathogenic effects (Kissoyan et al., 2019).

Under laboratory conditions, *C. elegans* are routinely cultured in a petri dish filled with solidified agar and seeded with the *Escherichia coli* (*E. coli*) B bacterial strain OP50 (Brenner, 1974). Neither the agar physical surface nor the OP50 bacteria is a replica of the natural environment encountered by the wild animal. In addition, a bleach procedure in eggs preparation kills all bacteria in the gut and surface of the worms, which precludes a vertical transfer of microbiome. However, the artificial environment (the OP50 and other artificial bacterial foods) and canonical strain (the Bristol strain N2) are an applicable and irreplaceable model to study the biological interactions of different species, as the majority of our knowledge on the animal comes from experiments with the N2 strain and its mutants cultured in a petri dish and fed with artificial foods. Thus, a better understanding of the effects of bacteria on *C. elegans* is critical for any study with this laboratory model. This is particularly important when the study is to assess the biological effects of a chemical, which is subjected to the metabolic effects both from the host and its bacterial food. In this regard, the *C. elegans* and its bacterial diet has become an alternative model to dissect the effect of

microbiome on the broader biological traits of the host (Garcia-Gonzalez et al., 2017; MacNeil, Watson, Arda, Zhu, & Walhout, 2013; Yilmaz & Walhout, 2016).

The chemotherapeutics 5-fluoro-2'-deoxyuridine (FUDR) is a commonly used drug in experiments with sterile worms. More recently, in the research that used the *C. elegans* and its bacterial diet model to study the host-bacteria interactions, it showed that the *E. coli* OP50 diet increases the drug efficacy in *C. elegans*, while the *Comamonas* diet (a genus of *Proteobacteria*) decreases it (Garcia-Gonzalez et al., 2017). The effect of the *E. coli* OP50 on the drug efficacy is dominant, as the diet with a diluted *E.coli* OP50 in *Comamonas* was still effective to potentiate the drug efficacy. The author reasoned that there are at least two possible mechanisms for the modulatory effects of the diet. One is that the OP50 passively supplements metabolites when the worms ingest it. The other is that there is an active mechanism by which the OP50 metabolizes the drug to increase its efficacy. Accordingly, they introduced two bacterial foods to differentiate these possibilities. One is the well-status live bacteria, and the other is the dead bacteria or bacteria power by disrupting the cells at high-pressure followed by lyophilization. With these bacteria-food tools and screening in a mutant bacteria library (3,985 strains from *E. coli* Keio collection) (Baba et al., 2006), the study established that the effect is dependent on active bacteria metabolism, leading to the novel discovery that genes involved in the RNA nucleotide metabolism in the OP50 modulate the efficacy of the drug FUDR in *C. elegans*.

In a previous study of the neurotoxicant methylmercury (MeHg) (Ke & Aschner, 2019), we hypothesized that the OP50 bacterial food could have a significant effect on the toxicity of MeHg, as MeHg itself in the aquatic environment is a product that is converted from inorganic mercury by some bacteria in sediments (Hamdy & Noyes, 1975). We investigated this by introducing different types of bacterial foods: live bacteria, dead bacteria, and dehydrated dead bacteria. Combing with our recent work on the chronic toxicity of MeHg with live bacteria (Ke et al., 2020), we concluded that live bacteria protects worms from toxic effects of a relative short exposure of MeHg (1–24 h), while a long term exposure (10 d) increases the toxic effects. By comparing the results from the three types of bacterial food, we were able to infer that both the active transport and passive diffusion of MeHg into the bacteria accounts for these effects (Ke & Aschner, 2019). In light of the clear advantages of these bacterial foods in the mechanistic studies with *C. elegans* in the areas of toxicology and pharmacology, it is essential to develop a robust protocol to produce different types of bacterial foods that are pertinent to the scientific questions of the study. Based on our own experiences (Ke & Aschner, 2019; Ke et al., 2020) and those from others (De Magalhaes Filho et al., 2018; Garcia-Gonzalez et al., 2017), herein, we developed a protocol on how to generate these useful bacteria-food based research tools.

Basic Protocol 1. Prepare concentrated *E. coli* OP50

There are at least three bacteria strains (OP50, Na22, and HT115) that are commonly used as a diet in a *C. elegans* lab. *E. coli* OP50 is a uracil auxotroph with limited growth rate on Nematode Growth Medium (NGM) plates, which is desirable for easier observation of worms. Na22 is a prototroph. Contrary to uracil-requiring OP50, the Na22 bacterial lawn is very thick, and suitable for growing large quantities of worms. The HT115 strain is the host

for plasmid RNAi clones, which is cultured on special RNAi NGM feeding plates that contain isopropylthio-β-galactoside (IPTG) and the ampicillin analog carbenicillin.

Here, we take the OP50 strain as an example to describe the steps to make a concentrated solution of freshly-made bacteria, which is a starting point for making a specific type of bacterial food.

Materials

Peptone (Fisher Scientific, BP1420–500)

Yeast Extract (Fisher Scientific, BP1422–500)

Agar (Fisher Scientific, BP1423–500)

Sodium Chloride (Fisher Scientific, S271–500)

Cholesterol (5 mg/ml in ethanol)

Nystatin (Sigma, C3045–25G)

$CaCl_2$ (Fisher Scientific, 349610250)

$MgSO_4$ (Sigma, M7506)

KH_2PO_4 (Sigma, P0662)

Streptomycin (Sigma, S9137)

Plastic weigh boat (Fisher Scientific, 02–202F)

Electronic scale (Fisher Scientific, 01-922-208)

100 mm petri dishes (Fisher Scientific, 08-757-100D)

Culture tubes (Fisher Scientific, 14-956-9C)

Narrow Mouth Erlenmeyer Flask, 3 L (Fisher Scientific, 09-841-129)

PYREX™ Reusable Media Storage Bottle, 1 L (Fisher Scientific, 06–423A)

Polygon Stir Bar (Fisher Scientific, 14-512-124)

Centrifuge with rotors for 50 ml tubes (Fisher Scientific, 75-333-839)

Flame Burner (Fisher Scientific, 03–962Q)

ddH_2O

Steps

A source for OP50 comes usually from a frozen stock solution with 15% glycerol stored in −80 °C freezer or liquid nitrogen tank, a saturated bacterial solution that was prepared recently, or a stab culture sent by the Caenorhabditis Genetics Center (CGC). To ensure that the bacteria culture is not contaminated with microorganisms from the environment during the process, the following steps should be performed aseptically.

1. Prepare liquid Lysogeny broth (LB): add the components (10 g peptone, 5 g yeast extract, 5 g sodium chloride, and 1000 ml ddH_2O) into a 1 L glass bottle. Autoclave and allow the solution to cool to room temperature. Add 1 ml (100mg/ml) streptomycin. Store the solution in a 4 °C freezer or cold room. The media can be stored at 4 °C for up to 1 year.
2. Prepare LB agar plates: add the components (10 g peptone, 5 g yeast extract, 5 g sodium chloride, 12 g agar, and 1000 ml ddH_2O) into a 3 L Erlenmeyer flask. Autoclave and allow to cool to 55 °C. Add 1 ml (100mg/ml) streptomycin. Dispense 25 ml of the solution into each 100 mm petri dish. After cool, store the plates in a 4 °C freezer or cold room. Freshly-made agar plates can be used for experiments within two months if they are stored in a plastic box at 4 °C.
3. Prepare NGM medium: add the components (3 g NaCl, 2.5 g peptone, and 975 ml ddH_2O) into a 1 L glass bottle. Autoclave and allow the solution to cool to 55 °C. Add 1 ml cholesterol (5 mg/ml in ethanol), 1 ml $CaCl_2$ (1 M), 1 ml $MgSO_4$ (1 M), 25 ml KH_2PO_4 (pH=6), 1 ml nystatin, and 1 ml 100mg/ml streptomycin. Turn off flame burner when pipette cholesterol, because ethanol is flammable. Store the solution in a 4 °C freezer or cold room. The media can be stored at 4 °C for up to 6 months.
4. Use a sterile loop to scrape some of the stock bacteria off of the top. Streak the loop across an LB agar plate. Invert and incubate the plates overnight at 37 °C.
5. Next day, prepare a pure culture of OP50 by inoculating a single colony from the plate into a 20 mL LB medium. Incubate the bacterial culture at 37 °C for 14–16 h in an incubator shaking at 220 rpm.
6. Next morning, centrifuge the bacterial culture at 2500 rcf for 10 min. Decant the clear supernatant. Add an equal volume of NGM medium. Vortex and centrifuge at 2500 rcf for 10 min. Repeat the process twice.
7. To make a 100× concentrated OP50, adjust the final volume of the bacterial culture to 200 μL with NGM medium. If experiment needs more bacterial food, multiple vials of bacteria culture can be prepared in step 5.

Basic Protocol 2. Titrate bacteria concentration

The procedure in basic protocol 1 is optimized to reduce the variation of bacteria concentration of each culture, yet quantifying bacteria concentration is necessary for the experiments that use a predefined concentration of bacteria or a mixture of different bacteria strains. The number of bacteria in a culture can be determined by estimation from the colony

numbers formed from a diluted bacterial solution. An alternative way to estimate the concentration of bacteria is by reading the absorbance of the bacterial solution at 600 nm. Here, we describe a method based on the optical density (OD) at 600 nm (OD600), to qualify concentration of a bacterial culture.

Materials:

Bacterial culture (see basic protocol 1, step 7)

NGM Media (see basic protocol 1, step 3)

Microplate reader (Molecular Devices, SpectraMax iD5 Multi-Mode Microplate Reader)

Clear bottom black 96-well plate (Fisher Scientific, 07-201-190)

Steps

1. To make a diluted bacterial solution, mix 1 volume of the concentrated bacteria from basic protocol 1 step 7 with 9 volumes of NGM medium.
2. Vortex and pipette 100 μL of the diluted bacterial solution into each of three wells per sample in a clear bottom black 96-well plate. Record the OD600 absorbance values of each well with a microplate reader. Use the mean value of the three wells for the bacteria concentration of the solution (bacteria concentration=$mean_{OD600} \times 8 \times 10^5$ cells/μl).
3. Repeat step 2 to get the desired concentration (OD600 value =1.00) of the bacterial solution by titrating the bacterial solution with NGM medium and concentrated OP50. For example, a freshly-made saturated OP50 bacterial solution usually has an OD600 value around 0.50.

Basic Protocol 3. Generate dead bacterial food by heating

Two types of dead bacteria can be produced by heating bacterial solution in a dry bath incubator at 80 °C for 1h (Ke & Aschner, 2019). One is the dead bacteria produced by heating 1 ml of harvested bacterial solution with a predetermined OD600 value. The other is the dehydrated dead bacteria produced by heating a 50 μL concentrated bacterial solution derived from 1 ml bacterial solution. To generate dead bacteria or dehydrated dead bacteria, it is important to begin with a predetermined concentration of live bacteria to make sure the number of bacterial cells among samples is equal. Please make sure all tubes that are used in the heating process must be sealed with parafilm to prevent evaporation or opening of the lid during the heating process.

Materials:

Bacterial culture (see basic protocol 2 step 3)

Heat incubator (Fisher Scientific, 88-860-021)

NGM Media (see basic protocol 1, step 3)

Steps

1. Aliquot the bacterial solution (OD600 value=1.00) from basic protocol 2 step 3 into 1.5 ml sterile Eppendorf tubes with 1 ml for each tube.
2. To generate dead bacteria, place one tube of the bacterial solution in a dry bath incubator at 80 °C for 1h.
3. To generate dehydrated dead bacteria, centrifuge one tube of the bacterial solution at 2500 rcf for 10 min. Decant the supernatant. Adjust the final volume to 50 μL. Vortex and incubate the tube in a dry bath incubator at 80 °C for 1h.
4. After heating, add 950 μL NGM medium into the tube to get 1 ml dehydrated bacterial food. Vortex to make a homogenized solution before feeding worms.

Basic Protocol 4. Generate dead bacterial food by antibiotics

The bacterial foods generated in basic protocol 3 can be used as a diet for worms on NGM plates or in NGM liquid. If one prefers to use plates seeded with a certain amount of dead bacteria, an alternative way to achieve this is to seed the plate with the OP50 bacteria (OD600=1.00) killed by kanamycin (50 μg/ml).

Materials:

Bacterial culture (see basic protocol 2 step 3)

NGM Media (see basic protocol 1, step 3)

Kanamycin (Sigma, K1377)

Peptone (Fisher Scientific, BP1420–500)

Agar (Fisher Scientific, BP1423–500)

Sodium Chloride (Fisher Scientific, S271–500)

Cholesterol (5 mg/ml in ethanol)

$CaCl_2$ (Fisher Scientific, 349610250)

$MgSO_4$ (Sigma, M7506)

KH_2PO_4 (Sigma, P0662)

Nystatin (Sigma, C3045–25G)

Streptomycin (Sigma, S9137)

Plastic weigh boat (Fisher Scientific, 02–202F)

Electronic scale (Fisher Scientific, 01-922-208)

35 mm petri dish (Corning, 430165)

Narrow Mouth Erlenmeyer Flask, 3L (Fisher Scientific, 09-841-129)

Polygon Stir Bar (Fisher Scientific, 14-512-124)

Flame Burner (Fisher Scientific, 03–962Q)

ddH_2O

Steps:

1. Prepare NGM plate: add the components (3 g NaCl, 2.5 g peptone, 17 g agar, and 975 ml ddH_2O) into a 3 L Flask. Autoclave and allow the solution to cool to 55 °C. Add 1 ml cholesterol (5 mg/ml in ethanol), 1 ml $CaCl_2$ (1 M), 1 ml $MgSO_4$ (1 M), 25 ml KH_2PO_4 (pH 6), 1 ml nystatin, and 1 ml 100mg/ml streptomycin. Turn off flame burner when pipette cholesterol, because ethanol is flammable. Swirl to mix well. Using sterile procedures to dispense the solution into 35 mm petri plates.
2. Mix 1 μl of 50 mg/ml kanamycin with 1 ml of the bacterial solution from basic protocol 2 step 3.
3. Pipette 25 μl of the bacterial solution into each 35 mm NGM plate.
4. Remove lid and place the plates in a fume hood for 15 min to dry the bacterial droplet on the plate.
5. After 14–16 h, check the viability of the bacteria with bacterial colony-forming unit (CFU) assay. To do this, the bacterial solution can be diluted by a factor of 10^6 with LB medium, and a certain amount (20 μl) of the diluted solution can be inoculated on a 100 mm agar plate (see basic protocol 1, step 4). With CFU assay, the number of bacteria can be calculated based on the number of bacterial colonies on the agar plate and the dilution factor. Adjust the concentration of kanamycin if there are viable bacteria.

Basic Protocol 5. Feed *C. elegans* with bacterial foods in liquid

C. elegans can be cultured both on agar and in liquid. In liquid culture, it is convenient to do high-throughput studies using 96-well plates. The advantage of liquid culture is that variations in bacteria concentration can be easily quantified. The following protocol follows the common step of synchronization of *C. elegans* (Stiernagle, 2006), and describes method for feeding *C. elegans* with bacterial food in NGM liquid.

Materials:

Bacterial culture (see basic protocol 2 step 3)

NGM medium (see basic protocol 1, step 3)

Larvae stage 1 (L1) *C. elegans*

5 N NaOH (Sigma, SX0603)

Household bleach (Clorox, 5% solution of sodium hypochlorite)

Stereo microscope (Zeiss, stemi 2000)

5-fluoro-2'-deoxyuridine (Sigma, F0503)

Sucrose (Sigma, S0389)

ddH_2O

Steps

1. Check the growth status of worms under a stereo microscope to make sure that the majority of worms are at gravid stage. For the wild type N2 worms, it is best to do synchronization on the third day (post 72 h) after chunking starved worms to a new plate.
2. Rinse the plate with 10~15 ml M9 briefly (<3 min). Don't soak the plate for an extended time to reduce the chance that you have to wash worms several more times to remove bacteria (once worms in liquid, they will thrash, thus moving out of the bacteria lawn).
3. Transfer M9 liquid from step 2 to a 15 ml tube, centrifuge at 1,800 rpm for 1 min. Remove the top liquid and add 10 ml fresh M9. Repeat the step twice to get a clear solution of worms.
4. Remove the top liquid, add 4~5 ml **FRESH** bleach solution (0.5 ml 5 N NaOH, 1 ml bleach, and 3.5 ml ddH_2O). Vortex briefly, place the 15 ml tube on a rotor for 3 minutes, then vortex for 15 seconds, and check the worms under microscope, to make sure majority of the worms have broken down and released eggs.
5. Add 10~12 ml M9. Vortex and centrifuge at 1,800 rpm for 1 min. Remove the top liquid carefully without disturbing the pellet. Add 10 ml fresh M9 and repeat the step twice.
6. Remove the top liquid, and add 9.5 ml **30%** sucrose. Vortex briefly, centrifuge at 2,800 rpm for 2 min.
7. Pipette a 4 ml from the top layer of the liquid (which includes the majority of eggs) into a new 15 ml tube, and add an equal volume of ddH_2O (4 ml). Vortex briefly and centrifuge at 1,800 rpm for 1 min.
8. Remove the top liquid as much as possible without disturbing the pellet. Add 10 ml ddH_2O to wash the eggs. Vortex briefly and centrifuge at 1,800 rpm for 1 min.
9. Remove the top liquid. Transfer eggs to an unseeded plate. To prevent eggs from forming aggregates, which are not good for hatching in some strains, pipette the solution droplet by droplet to avoid forming a large liquid area where there can be many aggregates.

10. Let the plate dry in a ventilator for about 10 minutes. Place the plate in a 20 °C thermostatic incubator.

11. After 16~18 hours, use NGM medium to harvest larvae stage 1 (L1) worms. Wash the worms twice with NGM medium by centrifugation at 1,800 rpm for 1 min.

12. Take a 50 μl sample of the worms. Pipette the sample by 8 to 10 droplets on a glass slide.

13. Count the total number of the worms under a stereo microscope (Stemi 2000, Zeiss). The mean number of the worms (worms/μl) is calculated with the total number divided by the volume. Calculate the total number of the worms harvested in the solution (total number = total volume × number of worms/μl).

14. Centrifuge worms at 1,800 rpm for 1 min. Decant carefully as much liquid without disturbing the worm pellet.

15. Mix the bacterial solution from basic protocol 2 step 3 with the worms (The volume of the bacterial solution is determined by the anticipated concentration of worms. Empirically the concentration of worms should be 10–20 L1 worms/μl depending on experimental conditions).

16. Load 100 μl worms with bacterial food per well into a 96-well plate. If a chemical treatment is considered, adjust the total volume of each well to 100 μl. Check the feeding status periodically to add bacteria when it is necessary. For example, chronic exposure experiments (10 days in liquid culture) usually need to add live OP50 bacteria (OD600=1.00~1.50) into treatment solution each time for every two days since day 4.

17. At 20 °C, place the plate in a wet chamber on a shaker at 200 rpm. To prevent evaporation, add adequate amount of ddH2O into the chamber each time for every 2 days.

Commentary

Background Information:

The methods developed herein aim to introduce the basic protocols for generating different types of bacterial foods, yet many novel types of bacterial food are being generated to address specific needs of experiments. For example, the bacteria OP50 can be disrupted by a homogenizer and lyophilized to generate bacteria powder (Garcia-Gonzalez et al., 2017), which can eliminate the effects from bacterial metabolism. Also, there are several libraries of mutant bacteria strains available for research (Baba et al., 2006; Watson et al., 2014), which provide extremely powerful tools to dissect mechanism of bacteria's effects on the biological traits of *C. elegans*. For the dead bacterial food, it is important to optimize the experimental conditions to generate *bona fide* dead bacteria, which can be achieved through checking viability of bacteria by inoculating the harvested bacterial solution and counting bacterial CFU on an antibiotic-based selective plate (Brugger et al., 2012).

For unfamiliar *C. elegans* strains treated in NGM liquid, a pilot study on the strain's characteristics should be carried out to avoid loss of the strain. Endpoints should include mutations, extra-chromosome arrays, allele balancers, segregation pattern, and optimal temperature for reproductive growth, growth rate, and numbers of viable eggs.

Lastly, to quantify the effect of bacterial foods, live bacteria and dead bacteria must be freshly made. For culturing bacteria in liquid medium, it often begins with inoculating a single colony from the agar plate that has been incubated in a 37 °C incubator overnight. For the freshly-made dead bacteria by heating, it is advisable to be used for experiments within 1 week if it is stored at 4 °C. Making a stock solution of the dead bacteria for an extended period could have issues that, during the freeze-thaw cycle, the physical and chemical properties of the stock solution are liable, which may introduce a potential bias. Carbenicillin is a preferred antibiotic for the HT115 strain as it can prevent the loss of plasmid RNAi clones (Maher, Catanese, & Chase, 2013). Moreover, the HT115 bacteria strain grows more slowly than the OP50 strain.

Critical Parameters and Troubleshooting:

To get a high quality of bacterial foods, always preparing a fresh culture is critical. It is important to make a freshly-cultured bacterial solution for each experiment, as in the post-log-phase growth period, the aging bacteria in old solutions is beginning to die. Old stock bacterial solutions inevitably contain some or otherwise significant amount of dead bacteria, which make the comparison of different bacterial foods inadequate. In addition, for experiments with liquid cultures, both the number of worms and concentration of bacterial foods have to be quantified. So the vortex step before sampling is important for an accurate measurement of both bacteria and worms. In step 15 of basic protocol 5, the volume of the bacterial solution should be significantly larger (at least 10 orders of magnitude) than the worm pellet, because if the volume of the bacterial solution is small, it will be diluted with the worm pellet. Several methods can be applied to overcome this difficulty when the concentration of bacteria must be strictly controlled. For example, when there is a significant change of bacteria concentration after mixing with worms, one can use the concentrated bacterial food and NGM medium from basic protocol 1 to titrate the solution. Moreover, the body size of L4 or adult-stage worms is significantly larger than the younger larvae, which requires a smaller number of worms (100 worms per well) for experiment using 96-well plate. If the worms are at late larvae stage, they can be picked and transferred into the bacterial solution.

Understanding results:

A concentrated bacteria solution (OD600>2.00) can be made from a 20 ml freshly-made saturated OP50 bacterial culture in LB medium (basic protocol 1). Although high OD600 values (>1.00) may not fit well linearly with the number of bacteria, if a highly concentrated bacteria solution is needed, bacterial solutions with a lower concentration can be enriched by centrifugation at 2500 rcf for 10 min following basic protocol 2. An alternative way is to use concentrated bacteria solutions (basic protocol 1) to feed worms, and this is particularly important when the experiment needs large quantity of worms.

For experiments with a relatively short-term treatment in NGM liquid (≤24 h), it is important to prepare enough bacterial food. A 20 ml saturated OP50 culture in LB medium can produce at least 5 ml bacteria with an OD600 value of 1.00. If the volume of a liquid culture in a well of 96-plate is 100 μl, a 20 ml saturated bacterial culture can be enough for 50 wells (basic protocol 5). For example, for 1,000 L1 stage worms (100 μl) cultured in a 96-well plate, a 20 ml saturated bacterial culture is enough for 50 groups with a period of 24 h.

For long-term treatment (≥24 h), live bacteria should be made freshly, so plan ahead to inoculate bacteria culture for experiment of next day. For example, for long-term treatment (such as 10 days) in 96-well plate, 200 L1 stage worms of each well can be fed with live bacteria (OD600=1.00) in the first 4 days without addition of the food, after which a 5 μl live bacteria (OD600=1.50) for each well can be added each time for every two days. To prevent hatching of eggs in liquid in long-term treatment, 50 μM FUDR can be added since day 4. Please note that some strains are very sensitive to the effect of FUDR (especially some knock-out strains and strains with GFP-tagged proteins expressed in neurons), many of which cannot pass the L3 stage and become dauer in liquid with FUDR. In these cases, early adult stage worms can be treated chronically in liquid.

In NGM liquid, live bacteria can proliferate, but dead bacteria cannot. When worms are needed to be fed with dead bacteria, a higher concentration should be added (basic protocol 5), and accordingly the quantity of the starting material, a freshly-made saturated bacteria culture, should be higher in amount than that for experiment with live bacteria (basic protocol 1). For short term treatment (≤24 h), dead bacteria derived from live bacteria (OD600=1.00) is enough for 24 h (basic protocol 3). For long-term treatment (such as 10 days) in 96-well plate, 200 L1 stage worms of each well can be fed with dead bacteria in the first 2 days without addition of the food, after which a 5 μl concentrated dead bacteria for each well (four times the concentration of the dead bacterial solution derived from live bacteria with OD600 of 1.00) can be added each time for every two days.

The growth speed of worms fed with dead bacteria is slower than worms with live bacteria. Though there is no apparent difference in the growth speed of L1 stage worms fed with dead or live bacteria in experiments of a short-term (≤12 h), the difference becomes significant when worms are treated for an extended time (≥24 h), for which the developmental speed should be taken into account when the study endpoints are influenced by developmental stage.

Time considerations:

LB, NGM medium, LB agar plates, and NGM plates take 1 day to prepare (basic protocol 1, and basic protocol 4). Inoculating from a stock bacterial solution to LB agar plate needs 1 day. Inoculating from a single bacterial colony to LB medium takes 1 day (basic protocol 1). It takes 3 days for the wild type N2 worms to develop into gravid stage after chucking an old plate with starved worms into a fresh OP50 seeded plate. However, if there are few worms in the old plate, it usually takes 6–7 days to get a populated young adult stage worms (basic protocol 5). It will take a longer time (~3 h) to titrate and measure concentration of bacteria at the beginning, but the time can be decreased significantly if one becomes familiar with the volume ratios of concentrated bacteria and NGM medium for certain values of OD600 (basic

protocol 2). The synchronization procedure (basic protocol 5) takes 1 h approximately. Counting and loading worms with bacterial food takes about 0.5 h.

Acknowledgments

This work was supported by the National Institutes of Health to MA (NIEHS R01ES007331 and R01ES10563).

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