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Bacterial Lawn Avoidance and Bacterial Two Choice Preference Assays in *Caenorhabditis elegans*

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[Abstract] Physical avoidance of pathogens is a crucial defense strategy used by the host to reduce pathogen infection. Hosts display the use of multiple strategies to sense and avoid pathogens, ranging from olfaction to sensing of damage caused by pathogen infection. Understanding various mechanisms of pathogen avoidance has the potential to uncover conserved host defense responses that are important against pathogen infections. Here, we describe protocols for studying pathogen lawn avoidance behavior as well as a change of bacterial preferences in the model nematode *Caenorhabditis elegans*. Besides, we describe the protocol for measuring preferences for pathogenic and nonpathogenic bacteria after training of the animals on pathogenic bacteria. These assays can be implemented in discovering various mechanisms of host learning that result in the avoidance of pathogens.

Keywords: Pathogen avoidance, Learning, Bacterial infection, Avoidance behavior, Defense response

[Background] A host uses multiple strategies to defend itself against pathogen infections (Medzhitov *et al.*, 2012). Physical avoidance of pathogens is one of the various defense strategies used by the host (Medzhitov *et al.*, 2012; Kavaliers *et al.*, 2019; Singh and Aballay, 2020). Different sensory mechanisms, including chemosensation and elicitation of pain by nociceptor neurons upon detection of bacterial toxins, lead to avoidance behaviors. A deeper understanding of the mechanisms leading to pathogen avoidance holds the potential to uncover conserved host defense responses that are important against pathogen infections.

The nematode *C. elegans* has been widely used to understand pathogen avoidance behavior and associative learning. *C. elegans* appears to use multiple mechanisms to learn about pathogens resulting in elicitation of avoidance behaviors (Singh and Aballay, 2020). *C. elegans* can sense bacterial metabolites (Tran *et al.*, 2017) as well as perturbations in core cellular activities (Melo and Ruvkun, 2012). Moreover, infection of the intestine can modulate neuroendocrine signaling to elicit avoidance behaviors (Singh and Aballay, 2019a and 2019b). Therefore, studying pathogen avoidance behavior and associative learning in *C. elegans* can help in deciphering various strategies used by a host in sensing pathogens.

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Materials and Reagents

1. 35 mm Petri dishes (Tritech Research, catalog number: T3500)
2. 60 mm Petri dishes (Tritech Research, catalog number: T3315)
3. 100 mm Petri dishes (Tritech Research, catalog number: T3361)
4. 14 ml polypropylene round-bottom tube (VWR, catalog number: 60819-761)
5. 15 ml centrifuge tube (VWR, catalog number: 21008-216)
6. 10 ml syringe (BD, Bacto™, catalog number: 309604)
7. 0.2 µm sterile syringe filter (VWR, catalog number: 28145-501)
8. *Escherichia coli* strain OP50 (Caenorhabditis Genetics Center (CGC))
9. *E. coli* strain HT115 containing control RNAi plasmid (L4440) (Source BioScience, Ahringer *C. elegans* RNAi Collection)
10. *E. coli* strain HT115 expressing dsRNA for any gene of interest (Source BioScience, Ahringer *C. elegans* RNAi Collection)
11. *Pseudomonas aeruginosa* strain PA14 (Liberati *et al.*, 2006)
12. *C. elegans* wild type Bristol N2 strain and/or any mutants of interest (CGC)
13. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Anatrace Product, catalog number: I1003 100 GM)
14. Sodium chloride (NaCl) (Fisher Bioreagents, catalog number: BP358-1)
15. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: 746495-500G)
16. Magnesium sulfate anhydrous ($MgSO_4$) (Mallinckrodt, catalog number: A31H10)
17. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P0662-2.5KG)
18. Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, catalog number: P3786-2.5KG)
19. Cholesterol (Sigma-Aldrich, catalog number: C8667-25G)
20. Ampicillin sodium salt (EMD Millipore, catalog number: 171254-25GM)
21. Bacto agar (BD, Bacto™, catalog number: 214030)
22. Bacto peptone (BD, Bacto™, catalog number: 211677)
23. Luria-Bertani (LB) broth (Sigma, catalog number: L3022-1KG)
24. 95% ethanol (EMD Millipore, catalog number: EX0280-3)
25. 5 mg/ml cholesterol (see Recipes)
26. 100 mg/ml ampicillin stock (see Recipes)
27. 1 M potassium phosphate buffer (pH 6) (see Recipes)
28. 1 M $CaCl_2$ (see Recipes)
29. 1 M $MgSO_4$ (see Recipes)
30. LB (see Recipes)
31. LB agar plates with and without ampicillin (see Recipes)
32. Nematode growth medium (NGM) agar plates (see Recipes)
33. RNAi plates (see Recipes)
34. Slow killing (SK) assay plates (see Recipes)

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Equipment

1. 500 ml conical flask (VWR, catalog number: 89091-420)
2. 1,000 ml conical flask (VWR, catalog number: 29136-106)
3. 2,000 ml conical flask (VWR, catalog number: 89090-858)
4. Pipetman (Eppendorf, models: P20, P1000)
5. 20 °C and 25 °C *C. elegans* incubators (ThermoForma, model: 3920)
6. Centrifuge (VWR, model: Clinical 50)
7. Platinum wire worm pick (made in the laboratory as described in Wollenberg *et al.*, 2013) and alcohol burner (VWR, catalog number: 470199-936)
8. Orbital Shaker (Thermo Fisher Scientific, catalog number: SK4000)
9. Autoclave (Consolidated Sterilizer Systems, model: SR-24D)
10. Stereomicroscope (Leica, model: MZ7.5)

Software

1. Microsoft Excel
2. GraphPad Prism

Procedure

A. *P. aeruginosa* Lawn Avoidance Assay

Day 1

1. Synchronization of *C. elegans*
   a. The assays are described for conditions where a gene is knocked down by RNAi (see Notes for details and assays with mutants). Transfer gravid adult N2 animals from *E. coli* OP50 plates to control RNAi plates (15-20 animals per RNAi plate), as well as RNAi plates for any gene of interest. Use one RNAi plate for each gene knockdown.
   b. Incubate the plates at room temperature (22 °C) for 2 h.
   c. Remove the gravid adults from the RNAi plates and incubate the plates at 20 °C for 72 h to obtain synchronized adult animals.

Day 2

2. Take out a vial of frozen *P. aeruginosa* PA14 from -80 °C and immediately streak on an LB agar plate. Incubate the plate at 37 °C for 12-14 h.

Day 3

3. Pick a single colony of *P. aeruginosa* PA14, inoculate in 2 ml of LB in a 14 ml polypropylene

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round-bottom tube, place vertically on a shaker, and grow it for 10-12 h at 250 rpm and 37 °C.

4. Place 20 µl of inoculum on the center of 35 mm SK plates (Figure 1A) that are modified NGM (3.5% instead of 2.5% peptone) plates. Let the inoculum dry at room temperature (20-30 min). Invert the plates upside down (to prevent cracks in agar from drying out) and incubate at 37 °C for 12 h.

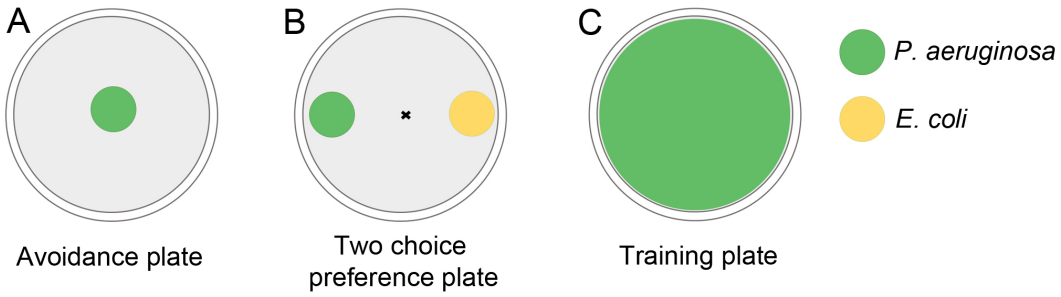

Figure 1. Schematic representation of plates. A. Avoidance assay plates contain a spot of pathogenic bacteria in the center. B. The two-choice preference assay plates contain spots of the two types of bacteria placed diagonally opposite. The cross (×) in the center of the two-choice preference assay plate indicates the site of the transfer of *C. elegans*. C. The surface of a training plate is fully covered with the pathogenic bacteria.

Day 4

5. Cool the *P. aeruginosa* plates from 37 °C to room temperature for 30 min.
6. Transfer 30 synchronized adult animals just outside *P. aeruginosa* lawn on SK plates. Use three *P. aeruginosa* plates per *C. elegans* strain/condition. Scrape off the residual *E. coli* from outside *P. aeruginosa* lawns on SK plates using platinum wire worm pick.
7. Incubate the plates with animals at 25 °C.
8. At various times of incubation (2, 4, 8, 12, and 24 h or as required), count the number of animals that are inside the lawn, outside the lawn, and have crawled on the sidewalls of plates.

B. Naïve Two Choice Preference Assay

Day 1

1. Synchronize *C. elegans* as described for lawn avoidance assay (Procedure A, Day 1) and incubate at 20 °C for 72 h.

Day 2

2. Take out a vial of frozen *P. aeruginosa* PA14 from -80 °C and immediately streak on an LB agar plate. Incubate the plate at 37 °C for 12-14 h.
3. Streak RNAi bacteria (*E. coli* HT115) from -80 °C on LB agar plates containing 100 µg/ml ampicillin. Incubate the plate at 37 °C for 12-14 h.

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Day 3

4. Pick a single colony of *P. aeruginosa* PA14, inoculate in 2 ml of LB in a 14 ml polypropylene round-bottom tube, place vertically on a shaker, and grow it for 10-12 h at 250 rpm and 37 °C.
5. Pick a single colony of RNAi bacteria (*E. coli* HT115), inoculate in 10 ml of LB with 100 µg/ml ampicillin in a 15 ml tube, place horizontally on a shaker, and grow it for 10-12 h on at 250 rpm and 37 °C.
6. After growth, concentrate RNAi bacteria 10-20 fold by centrifuging at 5,000 rpm for 5 min at room temperature.
7. Place 20 µl of each inoculum diagonally opposite onto 35 mm SK plates (Figure 1B). Let the inoculum dry at room temperature (20-30 min). Invert the plates upside down and incubate at 37 °C for 12 h.

Day 4

8. Cool the two-choice preference assay plates from 37 °C to room temperature for 30 min.
9. Transfer 30 synchronized adult animals to the centers of two-choice preference plates equidistant from both the lawns (Figure 1B). Use 3 two-choice preference assay plates per *C. elegans* strain/condition.
10. Scrape off residual *E. coli* from the center of the two-choice preference assay plates using a worm pick (as described in Procedure A, Day 4).
11. Incubate the plates with animals at 25 °C.
12. At various times of incubation (2, 4, 8, 12, and 24 h or as required), count the number of animals that are on the *E. coli* lawn, *P. aeruginosa* lawn, outside the lawns, and have crawled on the sidewalls of plates.

C. Trained Two Choice Preference Assay

Day 1

1. Synchronize *C. elegans* as described for lawn avoidance assay (Procedure A, Day 1) and incubate at 20 °C for 54 h.
2. Take out a vial of frozen *P. aeruginosa* PA14 from -80 °C and immediately streak on an LB agar plate. Incubate the plate at 37 °C for 12-14 h.

Day 2

3. Pick a single colony of *P. aeruginosa* PA14, inoculate in 2 ml of LB in a 14 ml polypropylene round-bottom tube, place vertically on a shaker, and grow it for 10-12 h at 250 rpm and 37 °C. Store the *P. aeruginosa* plate at 4 °C.
4. Place 20 µl of inoculum on 35 mm SK plates and spread it on the entire surface of the plates to obtain full lawns of *P. aeruginosa* (Figure 1C). Invert the plates upside down and incubate at 37 °C for 12 h.

5. Streak RNAi bacteria (*E. coli* HT115) from -80 °C on LB agar plates containing 100 µg/ml ampicillin. Incubate the plate at 37 °C for 12-14 h.

Day 3

6. Pick a single colony of *P. aeruginosa* from the plate stored at 4 °C on Day 2, inoculate in 2 ml of LB in a 14 ml polypropylene round-bottom tube, place vertically on a shaker, and grow it for 10-12 h at 250 rpm and 37 °C.
7. Pick a single colony of RNAi bacteria (*E. coli* HT115), inoculate in 10 ml of LB with 100 µg/ml ampicillin in a 15 ml tube, place horizontally on a shaker, and grow it for 10-12 h on at 250 rpm and 37 °C.
8. Cool the full lawn *P. aeruginosa* plates from 37 °C to room temperature for at least 30 min.
9. Transfer 54 h old synchronized *C. elegans* grown at 20 °C (from Day 1) to full lawns of *P. aeruginosa*, and incubate at 25 °C for 18 h. This will constitute the trained group of animals.
10. For control naïve animals, maintain the animals on their corresponding RNAi plates and incubate at 25 °C for 18 h along with the training groups.
11. Prepare two-choice preference plates (Figure 1B) as described above in naïve two-choice preference assay (Procedure B, Day 3). Invert the plates upside down and incubate at 37 °C for 12 h.

Day 4

12. Cool the two-choice preference assay plates from 37 °C to room temperature for 30 min.
13. Transfer 30 synchronized adult animals from the full lawn *P. aeruginosa* plates to the center of a two-choice preference assay plate equidistant from both the lawns. Use 3 two-choice preference assay plates per *C. elegans* strain/condition. This will be the trained group of animals.
14. Likewise, transfer 30 synchronized adult animals from RNAi plates to the center of a two-choice preference assay plate equidistant from both the lawns. Use 3 two-choice preference assay plates per *C. elegans* strain/condition. This will be the control group of naïve animals.
15. Scrape off the residual bacteria from the centers of two-choice preference assay plates using a worm pick (as described in Procedure A, Day 4).
16. Incubate the plates with animals at 25 °C.
17. After 1 h of incubation, count the number of animals that are on the *E. coli* lawn, *P. aeruginosa* lawn, outside the lawns, and have crawled on the sidewalls of plates.

Data analysis

Percent lawn occupancy calculation: Count the number of animals inside and outside the bacterial lawns. Calculate percent lawn occupancy in Microsoft Excel as:

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$$\text{Percent lawn occupancy} = \left(\frac{N_{on}}{(N_{on} + N_{off})}\right) \times 100$$

Where, N_{on} is the number of animals on the lawn, N_{off} is the number of animals outside the lawn. The animals that have crawled on the sidewalls of plates are excluded from calculations. Plot the time course of percent lawn occupancy using GraphPad Prism. The representative data in Figure 2A show that animals that have knockdown of the genes *aex-5* and *nol-6* have enhanced and slowed rates of avoidance of *P. aeruginosa* lawns, respectively.

Choice index calculation: Count the number of animals on the two types of bacterial lawns. Calculate *P. aeruginosa* choice index (*P. aeruginosa* CI) in Microsoft Excel as:

$$P.aeruginosa\ \text{CI} = \frac{[(\text{No. of worms on } P.aeruginosa) - (\text{No. of worms on } E.coli)]}{[(\text{No. of worms on } P.aeruginosa) + (\text{No. of worms on } E.coli)]}$$

The *P. aeruginosa* CI measures the preference of animals for *P. aeruginosa* with values ranging from -1 to 1. The values 1, -1, and 0 indicate that all animals are on *P. aeruginosa*, all animals are away from *P. aeruginosa*, and the same number of animals are on *P. aeruginosa* and *E. coli*, respectively. The *P. aeruginosa* CI can be plotted for single time points (trained two-choice preference assay) or for a time course (naïve two-choice preference assay) using GraphPad Prism. The representative data in Figure 2B show that naïve animals that have knockdown of the genes *aex-5* and *nol-6* have faster and slower switch in preference from *P. aeruginosa* to *E. coli*, respectively.

Use GraphPad Prism 8 for statistical analysis of data. Combine data from three independent experiments and calculate the mean and standard deviation (SD). Use multiple *t*-tests–one per time point and calculate p value for a gene knockdown with respect to the control sample. Judge the data to be statistically significant when $P < 0.05$.

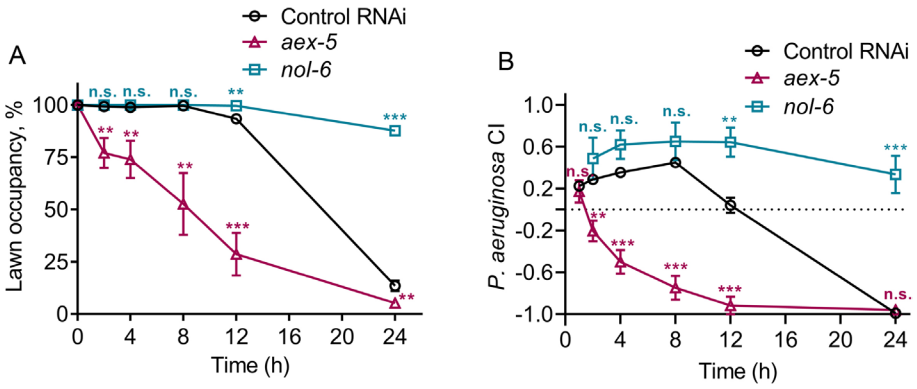

Figure 2. The time course of pathogen avoidance and change in bacterial preference. A. Time course of the percent occupancy of animals on *P. aeruginosa* lawns. B. Time course of the *P. aeruginosa* CI of naïve animals in a two-choice preference assay containing one lawn of each

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P. aeruginosa and *E. coli*. See Singh and Aballay (2019b), for details on *aex-5* and *nol-6*. Error bars denote SD from three independent experiments. *t*-test was used for each time point, and *P* values for a gene knockdown (*aex-5* or *nol-6*) were calculated with respect to the control RNAi. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. n.s., non-significant.

Notes

1. The assays are described for *P. aeruginosa*. However, these protocols can be used for other pathogens such as *Serratia marcescens* Db11.
2. The assays are described for conditions where a gene is knocked down by RNAi. The assays can be modulated for mutant animals where RNAi is not used. For such assays, carry out the synchronization of animals on *E. coli* OP50 plates and prepare lawns of *E. coli* OP50 and *P. aeruginosa* PA14 for two-choice preference assays.
3. The assays can be carried out at different levels of oxygen in a hypoxia chamber.
4. The incubation time of plates seeded with *P. aeruginosa* before animal exposure is critical for the kinetics of lawn avoidance. Differences in incubation time lead to different kinetics of avoidance (Singh and Aballay, 2019b).
5. There are several modifications of training protocol for two-choice preference assays. In some cases, training is carried out from L1 larval stage on plates that contain both *E. coli* and *P. aeruginosa* (Zhang *et al.*, 2005).
6. *P. aeruginosa* is an opportunistic pathogen, and the PA14 strain is a highly virulent clinical isolate from a human patient. Handling of *P. aeruginosa* PA14 should be carried out under biosafety level 2 (BSL-2) laboratory practices.

Recipes

1. 100 mg/ml ampicillin stock
   a. Add 1 g ampicillin sodium salt into a 15 ml tube and fill dH_2O up to 10 ml
   b. Stir until dissolved and filter-sterilize (0.22 µm)
   c. Store in 1 ml aliquots at -20 °C for up to 1 year
2. 5 mg/ml cholesterol
   a. Add 500 mg cholesterol to 100 ml 95% ethanol
   b. Stir until fully dissolved and filter-sterilize (0.22 µm)
   c. Store the cholesterol solution at room temperature
3. 1 M potassium phosphate buffer (pH 6)
   a. Add 23 g K_2HPO_4, 118 g KH_2PO_4 into a 1,000 ml graduated bottle and fill dH_2O up to 1,000 ml
   b. Stir until fully dissolved
   c. Keep bottle lid loose and autoclave for 30 min at 121 °C

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d. Store at room temperature

4. 1 M $CaCl_2$
   a. Dissolve 11.1 g $CaCl_2$ in 100 ml dH_2O
   b. Autoclave for 30 min at 121 °C
   c. Store at room temperature
5. 1 M $MgSO_4$
   a. Dissolve 12.0 g $MgSO_4$ in 100 ml dH_2O
   b. Autoclave for 30 min at 121 °C
   c. Store at room temperature
6. LB (100 ml)
   a. Add 2 g LB broth to 100 ml dH_2O in a 500 ml conical flask
   b. Autoclave for 30 min at 121 °C and store at room temperature
7. LB agar plates without ampicillin
   a. Add 10 g LB broth, 7.5 g agar to 500 ml dH_2O in a 1,000 ml conical flask
   b. Autoclave for 30 min at 121 °C and cool to 55 °C
   c. Pour 25 ml each into 100 mm Petri dishes and incubate at room temperature for 2 days
   d. Store at 4 °C in a box and use for 3 months
8. LB agar plates with ampicillin
   a. Add 10 g LB broth, 7.5 g agar to 500 ml dH_2O in a 1,000 ml conical flask
   b. Autoclave for 30 min at 121 °C and cool to 55 °C
   c. Add 500 µl of 100 mg/ml ampicillin while stirring
   d. Pour 25 ml each into 100 mm Petri dishes and incubate at room temperature for 2 days
   e. Store at 4 °C in a box and use for 3 months
9. Nematode growth medium (NGM) agar plates seeded with *E. coli* OP50
   a. Add 2.3 g Bacto peptone, 2.8 g NaCl, 20.4 g agar to 960 ml dH_2O in a 2,000 ml conical flask
   b. Autoclave for 30 min at 121 °C and cool to 55 °C
   c. Add 25 ml of 1 M potassium phosphate buffer, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and 1 ml of 5 mg/ml cholesterol while stirring
   d. Pour 8 ml each into 60 mm Petri dishes and incubate at room temperature for 3 days
   e. Inoculate a single colony of *E. coli* OP50 in 100 ml of LB broth in a 500 ml conical flask and incubate at 37 °C at 225 rpm shaking for 18-20 h
   f. Spot 400 µl of *E. coli* OP50 culture on the center of plates that were incubated at room temperature for 3 days (from step 9d)
   g. Grow *E. coli* OP50 on the plates for 3 days at room temperature
   h. Store the plates at 4 °C in a box and use for 3 months
10. RNAi plates (NGM plates with 3 mM IPTG and 100 µg/ml ampicillin)
    a. Add 2.3 g Bacto peptone, 2.8 g NaCl, 20.4 g agar to 960 ml dH_2O in a 2,000 ml conical flask
    b. Autoclave for 30 min at 121 °C
    c. Dissolve 715 mg IPTG in 5 ml dH_2O

d. Cool media to 55 °C while stirring
e. Add 25 ml of 1 M potassium phosphate buffer, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, 1 ml of 5 mg/ml cholesterol, 5 ml of IPTG solution prepared above (from step 10c), and 1 ml of 100 mg/ml ampicillin while stirring
f. Pour 8 ml each into 60 mm Petri dishes and incubate at room temperature for 3 days
g. Store the plates at 4 °C in a box and use for 3 months

11. Slow killing (SK) assay plates
    a. Add 3.2 g Bacto peptone, 3.0 g NaCl, 20 g agar to 960 ml dH_2O in a 2,000 ml conical flask
    b. Autoclave for 30 min at 121 °C and cool to 55 °C
    c. Add 25 ml of 1 M potassium phosphate buffer, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and 1 ml of 5 mg/ml cholesterol while stirring
    d. Pour 3.5 ml each into 35 mm Petri dishes and incubate at room temperature for 3 days
    e. Pack stacks of the plates in plastic bags, store at 4 °C in a box and use for 3 months

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Competing interests

The authors declare that they have no conflicts of interest or competing interests.

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