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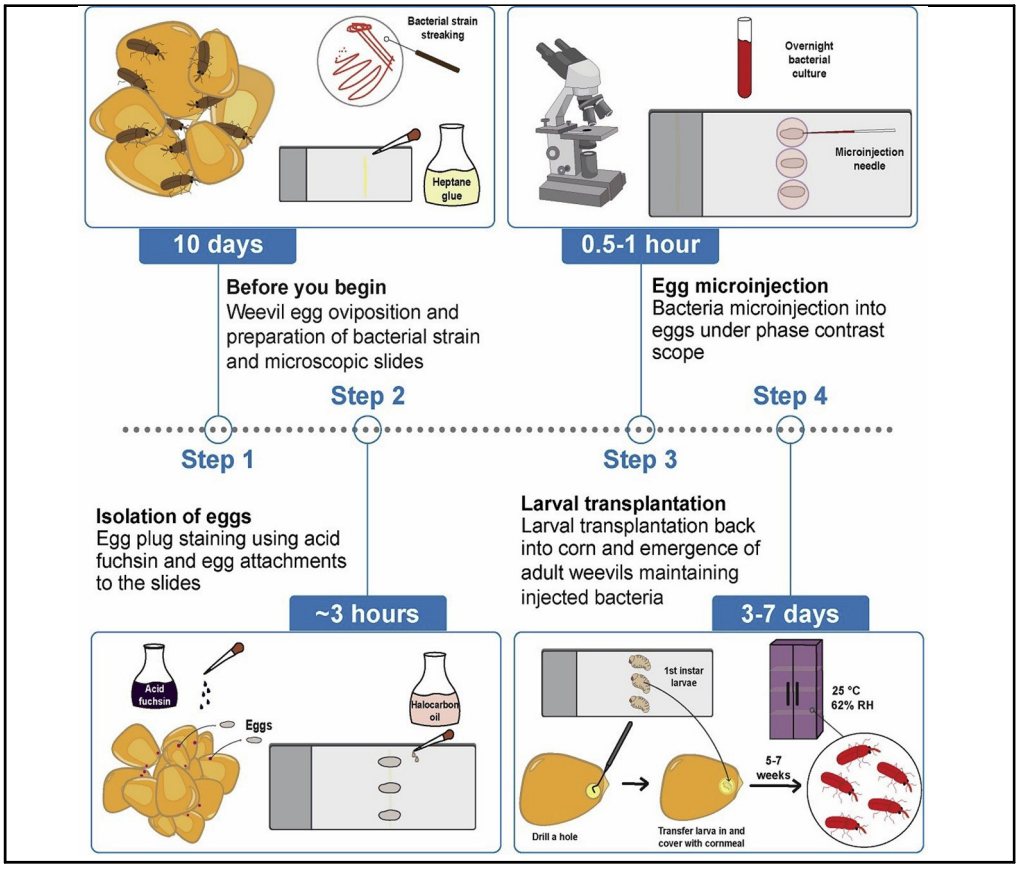
Protocol to establish a genetically tractable synthetic symbiosis between Sodalis praecaptivus and grain weevils by insect egg microinjection



We present a protocol to establish a synthetic symbiosis between the mCherry-expressing Sodalis praecaptivus and the grain weevil host, Sitophilus zeamais. We describe steps to isolate grain weevil eggs, followed by microinjecting the bacterial symbiont into insect eggs using a modified Drosophila injection protocol, which leads to localization of bacteria in female insect ovaries. We then detail larval transplantation and visualization of bacteria in live insects using a fluorescence dissection microscope to assess the transgenerational transmission to offspring in weevils.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for the establishment of synthetic symbiosis in grain weevils

Procedures to inject fluorescently labeled bacterial cells into grain weevil eggs

Steps for larval transplantation and visualization of bacteria in live insects

Provides a platform to study insect-bacterial interactions

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Protocol to establish a genetically tractable synthetic symbiosis between Sodalis praecaptivus and grain weevils by insect egg microinjection

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SUMMARY

We present a protocol to establish a synthetic symbiosis between the mCherry-expressing Sodalis praecaptivus and the grain weevil host, Sitophilus zeamais. We describe steps to isolate grain weevil eggs, followed by microinjecting the bacterial symbiont into insect eggs using a modified Drosophila injection protocol, which leads to localization of bacteria in female insect ovaries. We then detail larval transplantation and visualization of bacteria in live insects using a fluorescence dissection microscope to assess the transgenerational transmission to offspring in weevils.

For complete details on the use and execution of this protocol, please refer to Su et al. (2022).[1]

BEFORE YOU BEGIN

The association between the grain weevil, Sitophilus zeamais and its bacterial symbiont, Sodalis pierantonius, is characterized as a recently derived (nascent) symbiosis.[2] It therefore represents an excellent system to study early adaptations in symbiosis and to serve as a platform for experimental symbiont replacement. In this protocol, we explain how a closely-related, free-living relative of the grain weevil symbiont, named Sodalis praecaptivus, can be established as a transovarially-transmitted synthetic symbiont, yielding a novel platform for experimentation in symbiosis.

In contrast to microinjection of Drosophila[3] and mosquito[4] eggs, which are well established and relatively straightforward, grain weevils have a complex life cycle that mandates intensive husbandry in the microinjection and rearing procedures. Grain weevils oviposit around 400 eggs over their lifespan (6–8 months) and females drill cavities in maize grains and oviposit one egg per cavity with a transparent egg plug sealing the entrance to prevent dehydration.[5] Eggs often require 4–7 days to hatch under laboratory rearing conditions (25C, 60%), and larvae subsequently feed only on grain endosperm and emerge as adults following completion of metamorphosis in 5–7 weeks. Their eggs are very fragile as a consequence of a thin surrounding chorion (shell), making them susceptible to mechanical damage and dehydration during extraction and manipulation. This protocol describes a number of specialized manipulation and husbandry steps, established through extensive trial and error, to overcome these inherent challenges and derive an egg microinjection and rearing protocol with a relatively high level of success.

In order to better focus on the distribution of the injected S. praecaptivus following egg microinjection, all procedures shown in this protocol used a mCherry expressing strain of S. praecaptivus, designated as strain MC1, allowing us to visualize these bacteria in live insects using a fluorescence dissection microscope.

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Maximization of weevil egg oviposition

Timing: 10 days

Under optimal conditions, grain weevils lay one egg per grain.[6] Therefore, it makes sense to rear them on a large quantity of maize in the lab to ensure availability of a large number of offspring for weevil stock maintenance. For a general description of weevil husbandry in the laboratory, see Su et al.[1] However, inspection of a large number of kernels presents a significant burden in terms of time and eggs need to be injected within 24 h post oviposition. Therefore, when harvesting eggs for this procedure, it makes sense to decrease kernel quantities to enforce them lay more eggs in each kernel instead of distributing eggs in multiple kernels, maximizing the efficiency of egg discovery and subsequent extraction.

1. Hydrate the organic whole yellow maize kernels (Purcell Mountain Farms) with deionized (DI) water (3%; w/v).
2. Transfer 200–300 young adult weevils from a stock culture into a new rearing container with 20–30 maize grain kernels. All weevils are reared in Darwin insect chamber at 25C and 62% relative humidity (RH).
3. Replace with fresh maize grains every 2 days for a week and then everyday for 5–7 days.

   Note: We find that weevils are most productive in egg laying when grains are replaced frequently.

   ⚠ CRITICAL: When replace maize grains the day before egg injection, calculate the time backward carefully because we only allow weevils to oviposit for 18–20 h prior to egg collections to ensure we inject the eggs at early embryonic stage.

Injection slide preparation

Timing: 20 min

4. Use a ruler and a marker to draw a line that is perpendicular to the long side of the microscope slide.
5. Flip the microscope slide upside down for glue attachment.
   a. Dip a 1,000 mL pipette tip in heptane glue solution allowing the pipette tip to suck up around 50 mL of glue by capillary action.

   Note: Heptane glue is obtained by soaking a large amount of double-sided tape in heptane for one day to create a saturated heptane glue solution. (However, the saturated heptane glue solution can be diluted as needed for the procedure).

   b. Leave the slides with heptane glue to air dry for at least 15 min before use.
6. Store all injection slides in a slide storage box with dividers to prevent them from sticking together.

Bacterial strain preparation

Timing: 2 days

7. Streak S. praecaptivus MC1 from 80C freezer on LB agar plate with IPTG (100 mM) and X-gal (40 mg/mL) and incubate at 30C for 2 days before use. A fresh plate is required to make an over-night culture for microinjection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Sitophilus zeamais (female and male; all life stages)	USDA, Manhattan, KS, U.S.A. https://www.ars.usda.gov/plains-area/mhk/cgahr/	N/A
Drosophila melanogaster (genotype: y w; female and male; all life stages)	Obtained from the Golic lab, School of Biological Sciences, University of Utah, U.S.A.	N/A
Bacterial and virus strains		
S. praecaptivus MC1	Su et al.[1]	CD 2555
Chemicals, peptides, and recombinant proteins		
Halocarbon oil 700	Sigma-Aldrich	H8898-50ML
Acid Fuchsin	Thermo Scientific	AC400210250
Acetic acid	Sigma-Aldrich	64-19-7
IPTG	Genesee Scientific	20-109
X-gal	Genesee Scientific	20-108
Heptane	Sigma-Aldrich	HX0090
Tryptone	Sigma-Aldrich	T7293
Agar	Sigma-Aldrich	05039
Yeast extract	Sigma-Aldrich	Y1625
Deposited data		
S. praecaptivus MC1	NCBI sequence read archive (SRA)	SRA:SAMN26947704
Software and algorithms		
Adobe Illustrator	Adobe Inc.	https://www.adobe.com/products/illustrator.html
Other		
Capillary tubes	Drummond	#2-00-203-G/X
Needle puller	Sutter Instrument Co	Model P-97
Microscope glass slides	Bio Optica	09-1000MB
Spectrophotometer	DiluPhotometer	OD600
Fluorescence microscope	Leica	M205
Organic whole yellow maize kernels	Purcell Mountain Farms	N/A
Syringe	Sigma-Aldrich	Z248029
Micromanipulator	Narishige	M-152
Glutinous rice flour	Erawan Brand	N/A
Phase contrast scope	Zeisis	872E
De-chorionator	Cole-Parmer	UZ-06287-18

MATERIALS AND EQUIPMENT

LB agar plate

Reagent	Final concentration	Amount
Tyrptone	1% (w/v)	5g
Yeast extract	0.5% (w/v)	2.5g
Agar	1.5% (w/v)	7.5g
Deionized (DI) water	N/A	500 mL
IPTG (100 mM)	100 mM	500 mL
X-gal (100 mg/mL)	40 mg/mL	200 mL
Total	N/A	500 mL

Store in the refrigerator (4C–8C) for up to 3 weeks.

Acid fuchsin		
Reagent	Final concentration	Amount
Acid fuchsin	0.35% (w/v)	1.75 g
Acetic acid	25% (v/v)	125 mL
Deionized (DI) water	N/A	375 mL
Total	N/A	500 mL
Store in room temperature for 4–6 months.		

STEP-BY-STEP METHOD DETAILS

Isolation of weevil eggs

Timing: 3 h

This section describes how to localize eggs inside maize grains and how to extract and transfer eggs on microscope slides in preparation for injection.

1. Egg plug staining using acid fuchsin:
   a. Make acid fuchsin by adding 1.75 g acid fuchsin and 125 mL acetic acid to 375 mL of deionized (DI) water.[6,7]
   b. Within 24 h following oviposition, soak grains harboring weevil eggs in deionized (DI) water for 5 min before staining.
   c. Add 5 mL acid fuchsin to the resulting hydrated grains and then allow the gelatinous egg plugs to stain for 1 min.
   d. De-strain the grains in DI water 10 times until egg plugs can be readily distinguished by their higher intensity of pink staining (Figure 1A).
   e. Remove the egg plugs using fine forceps under the dissection scope, and the egg inside each cavity is also carefully removed for use in the microinjection procedure using tweezers or a de-chorionator (Figure 1B).
2. Egg preparation for microinjection.
   a. Attach isolated eggs in a consistent polar orientation to the microscope slide with heptane glue (described in "before you begin") to preclude the possibility of egg movement during microinjection.
   b. Following attachment, dehydrate eggs for 5 min at 25C.

⚠ CRITICAL: Presence of wrinkles on the egg surface is indicative of excessive dehydration or damage resulting from handling. These eggs do not yield a favorable outcome and should be discarded.

   c. After dehydration, place 2 mL drop of gas-permeable halocarbon oil on the surface of each egg yielding complete immersion, to prevent subsequent dehydration.

Egg microinjection

Timing: 0.5–1 h

This part explains how to prepare and set up for weevil egg microinjection.

3. Needle preparation for microinjection.
   a. Inoculate one bacterial colony from a fresh plate culture of S. praecaptivus MC1 strain into 3 mL of LB media and let the suspension grow in a 30C shaking incubator (200 rpm) for 17–20 h.
   b. Concentrate the bacterial culture to OD_{600nm} = 1 in sterile bacteriological saline (0.85% NaCl).

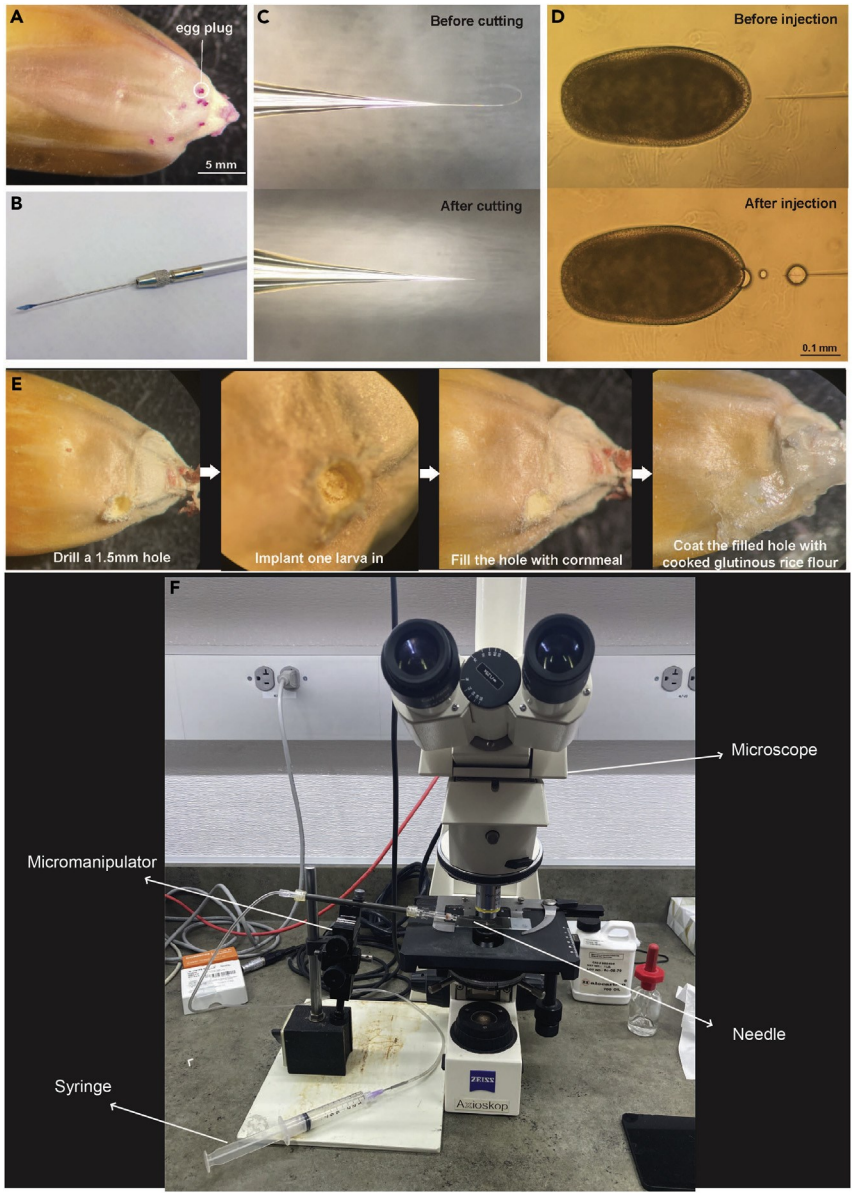

Figure 1. Demonstration of weevil egg microinjection procedures

(A) Acid fuchsin stains egg plugs with a pink color visible on the surface of maize grains and the white circle highlights one example.

(B) The de-chorionator proves to be a useful tool in the egg isolation step.

(C) Before (top) and after (bottom) shots of an injection needle undergoing breakage to provide an appropriate injection aperture.

(D) Before (top) and after (bottom) shots of the egg microinjection, showing a typical level of leakage of fluid/tissue following injection.

(E) Demonstration of larval transplantation procedure (ordered from left to right).

(F) Injection system composed of phase contrast microscope, syringe, micromanipulator, and a needle.

c. Add 2 mL of the prepared bacterial suspension to one end of a 3.5^{00} glass capillary tube (Drummond #2-00-203-G/X) via capillary action prior to pulling and then pull the tubes in a needle puller with settings of heat = 270, pull = 20, velocity = 40 and time = 150.

d. Under a dissection scope, use sterile forceps to break the tip of the needle, yielding a sharp orifice with no curvature in the needle (Figure 1C).

4. Egg microinjection under phase contrast scope (Figure 1F).
   a. Attach the prepared needle to an empty syringe held by a micromanipulator (Narishige, Model M-152) to facilitate accurate subsequent injection.
   b. Push the syringe to test if the needle is open at the sharp end following breakage.

   Note: If the liquid in the needle can be pushed to the tip, then it is ready to use. If not, return to the previous step and create another break.

   c. Once the needle is ready to use, the needle needs to be set up with the tip pointing down at an angle of 30 degrees.
   d. Arrange the needle to be perpendicular to the line of eggs on the injection slide.
   e. Before loading the microscope slide, adjust the needle position using the manipulator to focus the needle under the phase contrast scope.
   f. Prepare a microscope slide with a drop of halocarbon oil on the stage. Immerse the tip of needle in the oil and adjust the needle position again.
   g. Roll down the stage and load the injection slide on the stage. Move the stage up bringing the eggs to the same level as the needle.

   Note: Ensure both the needle and the edge of the eggs are in focus before injection.

   h. Perform injections into the posterior pole of the eggs by moving the slide towards to the needle (Figure 1D).

   Note: Approximately 0.005–0.02 mL of bacterial culture is then injected into each egg, containing an estimated 800–1,000 number of bacterial cells.

   ⚠ CRITICAL: A small drop of liquid is commonly observed adjacent to the posterior pole of the egg after removing the needle, as shown in Figure 1D. However, if a larger volume of liquid is observed to leak from the egg, it is often indicative of excessive damage to the chorion, leading to failure of the procedure.

   i. Following injection, maintain the slide in an incubator at 25C and 62% RH for 3–7 days until larvae hatch.

Larval transplantation

Timing: 3–7 days

This section addresses how to handle newly hatched 1st instar larvae and continue rearing them to adult stage, as depicted in Figure 1E.

⚠ CRITICAL: First instar larvae need to be transferred back into grain within 24 h after hatching.

5. Prepare maize grains for larval transplantation.
   a. Soak maize grains in deionized (DI) water for 5 min to facilitate weevil transplantation and subsequent survival.
   b. Drill a 1.5 mm diameter hole in the narrow end of the maize kernel.
6. Transfer larva to the hole.
   a. Gently touch the larva under the dissection scope using the smooth side of the tweezer tip and watch for movement, signifying viability.

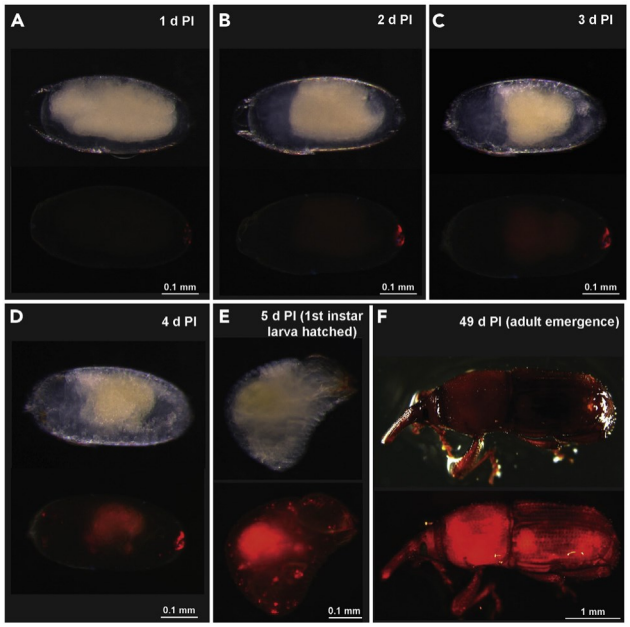

Figure 2. Microinjection of S. praecaptivus MC1 in grain weevils

The same specimens are depicted under normal light (upper) and mCherry fluorescence (lower).
(A) Egg at one day post injection (PI) with infection observed at the injection site (posterior pole of the egg).
(B–D) Eggs at 2–4 days post injection (PI), with bacteria proliferating inside the egg.
(E) First instar larva following hatching at five days post injection, with dense infection in the developing gut.
(F) Adult weevil, injected at egg stage, following emergence from grain. Figure reprinted with permission from Su et al.[1]

b. Gently roll the larva on the microscope slide to remove the halocarbon oil.
c. Carefully implant a single larva into the hole in maize prepared as described above.
d. Gently fill the hole, embedding the larva, with powdered cornmeal.

7. Coat the filled hole with glutinous rice flour solution.
   a. Make the glutinous rice flour solution by adding 0.5 g glutinous rice flour to 2 mL DI water and heating the solution in microwave to boil for 30 s. This provides sufficient material to seal 30 holes.
   b. Seal the hole with a light, transparent layer of cooked glutinous rice-water mixture, simulating the protective coating that is found on the grain surface.
8. The transplanted grains are then maintained under standard rearing conditions (25C, 62% RH) for 5–7 weeks to facilitate emergence of adult weevils.

EXPECTED OUTCOMES

Weevil egg microinjection

In order to demonstrate the colonization and maintenance of bacteria in grain weevils following injection into eggs, daily monitoring was performed using a fluorescent dissection microscope (Leica M205 FCA; exposure: 861 ms, gain: 3), shown in Figure 2. Following injection of S. praecaptivus MC1 into the posterior pole of the egg (Figure 2A), bacteria proliferate in concert with weevil embryonic development (Figures 2B–2D) and achieve dense infection in the resulting larval (Figure 2E) and adult stages (Figure 2F).

To better illustrate the efficiency of this procedure, the weevil survival rate for each step is presented in Table 1, using a batch of injection composed of 96 weevil eggs, and the bacterial infection rate in the resulting adults is 100%. A separate experiment was performed to quantify the number of

Table 1. Survival rate of grain weevil egg microinjection procedures

	Step 1: Isolation of weevil egg	Step 2: Egg microinjection	Step 3: Larval transplantation
Alive	38	29	6
Dead	58	29	29
Survival rate	40%	50%	20%

Data re-used with permission from Su et al.[1]

bacteria reside in larval and adult weevils, revealing an averaged bacterial density of 3.74×10^4 CFU per 1st instar larva (n = 5; SD = 2.88×10^4) and 7.95×10^6 CFU per adult (n = 5; SD = 2.19×10^6).

Potential utility in other insect hosts

Microinjection of S. praecaptivus MC1 was also performed in Drosophila melanogaster eggs following the well-established Drosophila egg injection protocol.[2] When injection was conducted using the concentration of bacterial culture described above and established as appropriate for weevils, it proved to be deleterious to Drosophila embryonic and larval development, resulting in a high level of mortality. However, injection of a 100-fold diluted suspension led to the successful establishment of infection throughout larval development and subsequent infection of adults with no notable deleterious impacts. The resulting mean bacterial concentration was found to be 1.15×10^7 CFU per adult (mean; n = 4). Figures 3A and 3B demonstrates the presence of mCherry-expressing S. praecaptivus in Drosophila adults following microinjection at egg stage. Dissections and inspection of the resulting Drosophila female ovaries were performed (Figure 3C) and the further confocal imaging revealed the presence of S. praecaptivus around the region of germline stem cells (Figure 3D). However, to date, no offspring have been obtained that are found to maintain the bacteria. This could be a consequence of (i) elimination of the bacteria in subsequent stages of embryonic or larval development or (ii) death of embryos or larvae that maintain the bacteria (consistent with the pathological effects observed when eggs were first injected). Clearly, further experimentation is necessary to understand the trajectory of infection and transmission in D. melanogaster. However, given that Drosophila spp. and grain weevils are only distantly related and that these fruit flies are not known to harbor Sodalis-allied symbionts, we consider these results to be relatively encouraging in terms of applying this technique towards other insect hosts.

LIMITATIONS

The main limitation for this egg microinjection protocol in weevils is the efficiency of obtaining live adults. Many steps in the procedure mandate careful handling of delicate eggs and young larvae. However, following establishment, resulting lines of insects have been maintained through 12 generations of transovarial transmission (to date and ongoing) through selection of offspring demonstrating mCherry fluorescence.

TROUBLESHOOTING

Problem 1

Eggs fail to hatch to 1st instar larvae following injection of bacteria.

Potential solution

The main cause for this failure is injury to the egg incurred during isolation and subsequent injection steps (steps 2 and 4). It can be helpful to practice extraction of eggs and rearing of larvae on the slide in the absence of injection to improve these handling procedures.

Problem 2

The egg burst or leaks large amount of fluid/tissue immediately after the injection needle enters the egg (Egg microinjection step).

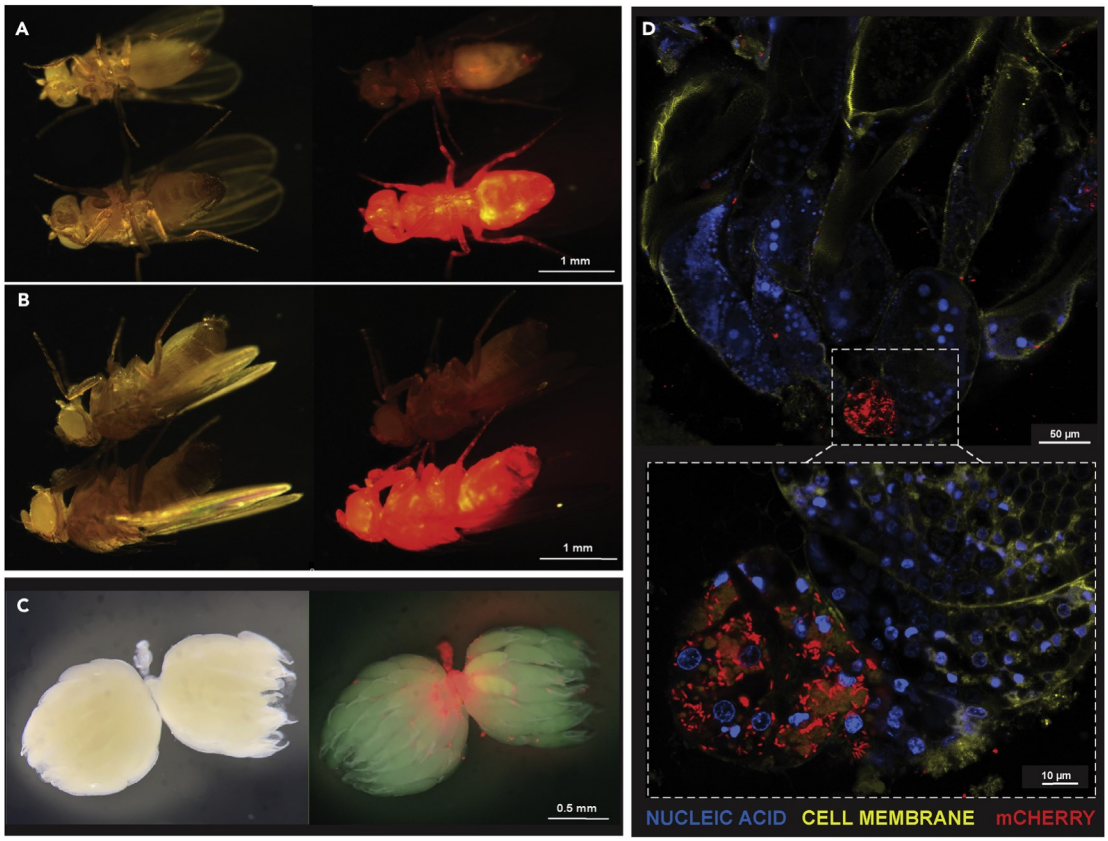

Figure 3. Microinjection of S. praecaptivus MC1 in Drosophila

(A–C) each depict an identical specimen illuminated under normal light (left) and mCherry fluorescence (right). (A) Ventral view and (B) lateral view of a non-injected Drosophila adult (top) next to a Drosophila adult injected with S. praecaptivus MC 1 at egg stage (bottom), showing intense mCherry fluorescence throughout the body. (C) Ovaries from a mated Drosophila female derived from a microinjected egg.

(D) Confocal imaging of Drosophila ovarioles. S. praecaptivus MC1 infection is present in the anterior region of the ovariole clusters, which could be the terminal filament and cap cells or the germline stem cells. Specimens were stained with Hoechst 33342 (blue: targeting nucleic acid) and CellMask Green (yellow: targeting cell membranes).

Potential solution

This can result from three procedural errors. First, the needle might be cut in such a way that its orifice is too large, creating a large hole/rupture in the egg upon entry (step 4). As outlined in the protocol, it makes sense to obtain a needle with the smallest possible diameter orifice, ensure that it is open to liquid passage and increase the size gradually until it works. A second possibility is that the egg is insufficiently dehydrated. Note that our laboratory is located in Utah, U.S.A., where relative humidity is typically very low, likely enhancing dehydration. Use of this procedure in a more humid environment might entail adoption of a longer period of dehydration. The third possibility is that excessive pressure is applied to the egg upon transfer to the slide. Use the minimum amount of pressure possible to attach the egg to the heptane glue. Again, this step can be practiced and optimized in the absence of injection.

Problem 3

Adults fail to emerge following larval transplantation into maize.

Potential solution

This can occur as a consequence of death following injection and for that reason it is important to ensure that larvae are alive/active following injection (step 5). In addition, the small, young larvae can easily be damaged with excessive force from forceps. To counter this problem, forceps can

be modified by application of a drop of epoxy resin on one interior arm, such that it precludes their complete closure, yet retains sufficient purchase to grasp the larva.

Problem 4

An egg injected with Sodalis praecaptivus yielded a non-infected weevil adult.

Potential solution

The most likely reason for this is the failure of the injection procedure to deliver bacterial cells into the egg. There are two mistakes that are easy to make: (1) the needle does not penetrate the egg during the injection procedure (step 4). This can happen because the egg surface is very soft and slippery in the halocarbon oil and the needle can slide over the egg surface while appearing to have achieved entry. It is important to make sure that the needle and the embryo are in focus and on the same plane under the phase contrast scope. (2) When injection is performed manually, it is very difficult to control the exact volume of the bacterial culture being injected. In addition, micro-injection of liquid culture into an egg is countered by the pressure in the interior of the egg, often yielding different levels of fluid/tissue leakage. If the injected volume is too low, the internal pressure can push the bacterial cells out, leading to emergence of a non-infected adult. This problem can be difficult to address but often improves with experience (step 4).

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Colin Dale (colin.dale@utah.edu).

Materials availability

The bacterial strain utilized in this study is available upon request from the lead contact, Colin Dale (colin.dale@utah.edu).

Data and code availability

- The accession number for the sequence reads derived from Sodalis praecaptivus MC1 reported in this paper is SRA: SAMN26947704.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Y.S., H.L., and C.D. developed the protocol. Y.S. and C.D. analyzed data, wrote the manuscript, and prepared figures. All authors read and provided edits for the manuscript and agree to its contents.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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