Honey bee functional genomics using symbiont-mediated RNAi

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Abstract

Honey bees are indispensable pollinators and model organisms for studying social behavior, development, and cognition. However, their eusociality makes it difficult to use standard forward genetic approaches to study gene function. Most functional genomics studies in bees currently utilize double-stranded RNA (dsRNA) injection or feeding to induce RNAi-mediated knockdown of a gene of interest. However, dsRNA injection is laborious and harmful, and dsRNA feeding is difficult to scale cheaply. Further, both methods require repeated dsRNA administration to ensure a continued RNAi response. To fill this gap, we engineered the bee gut bacterium *Snodgrassella alvi* to induce a sustained host RNA interference response that reduces expression of a targeted gene. To employ this FUGUES (**FU**nctional **G**enomics **U**sing **E**ngineered **S**ymbionts) procedure, a dsRNA expression plasmid is cloned in *Escherichia coli* using Golden Gate assembly and then transferred to *S. alvi*. Adult worker bees are then colonized with engineered *S. alvi*. Finally, gene knockdown is verified through qRT-PCR, and bee phenotypes of interest can be further assessed. Expression of targeted genes is reduced by as much as 50-75% throughout the entire bee body by five days after colonization. This protocol can be accomplished in four weeks by bee researchers with microbiology and molecular cloning skills. FUGUES currently offers a streamlined and scalable approach for studying the biology of honey bees. Engineering other microbial symbionts to influence their hosts in ways that are similar to those described in this protocol will likely lead to useful methods for studying additional insect and animal species in the future.

Related links
Key references using this protocol

Leonard, S. P. *et al.* Engineered symbionts activate honey bee immunity and limit pathogens. *Science* **367**, 573–576 (2020).

Leonard, S. P. *et al.* Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synth. Biol.* **7**, 1279–1290 (2018).

Introduction

The western honey bee, *Apis mellifera*, supports global agriculture and is a model organism for studies of insect cognition, sociality, and gut microbiomes. Despite this widespread use and economic importance, our understanding of honey bee genetics is underdeveloped because it is difficult to breed and maintain many isolated colonies of this and other eusocial bee species. Most studies to date have examined differences in phenotypes due to natural genetic variation or how gene expression changes after an experimental treatment[1–4].While these studies can suggest candidate genes involved in different biological processes and pathways, they are correlative and cannot rigorously establish that changes in gene expression cause a change in behavior or other phenotypes. Disrupting or altering the expression of a target gene and observing the corresponding phenotypic effects is a fundamental approach for establishing gene function.

RNA interference (RNAi) is a highly conserved antiviral immune mechanism that has been exploited to suppress gene expression and investigate gene function in many organisms, including insects, nematodes, and plants[5–8]. The RNAi pathway is activated by the presence of double-stranded RNA (dsRNA) in cells. Enzymes, including Dicer, cleave dsRNAs into shorter ~21 nucleotide fragments which are loaded onto the RNA-induced silencing complex (RISC). RISC degrades or impedes translation of RNAs with sequences that match or are similar to these fragments, reducing expression of any genes they encode. Researchers have induced targeted RNAi responses in various organisms by injecting or feeding purified dsRNA, or by feeding them dsRNA-producing *Escherichia coli* cells[8]. Persistent induction of RNAi has also been achieved in insect pest species by engineering natural gut symbionts to stably express dsRNA[9].

RNAi can be used to knock down expression of a specific gene and observe how this affects organismal phenotypes in order to study the function of that gene. However, injecting bees with dsRNA to induce RNAi is labor intensive and traumatic, and feeding dsRNA to bees has been reported to have mixed success at eliciting an effective RNAi response. These shortcomings inspired us to test a different delivery modality in which we express dsRNA from a bacterium that is normally present in the honey bee gut microbiome. We recently showed that this symbiont-mediated RNAi approach can be used to robustly, persistently, and systemically knock down gene expression in bees[10]. We refer to this approach as FUGUES, for **fu**nctional **g**enomics **u**sing **e**ngineered **s**ymbionts. Here we provide a protocol for performing FUGUES to establish connections between genes and phenotypes in adult worker honey bees.

Overview of the procedure

We provide a detailed, step-by-step protocol for implementing FUGUES in honey bees through symbiont-mediated RNAi (Fig. 1). First, a dsRNA expression vector targeting an *A. mellifera* gene of interest is designed, assembled, and transformed into an *E. coli* cloning strain. Next, the dsRNA expression vector is transferred to the bee symbiont, *Snodgrassella alvi*, and honey bees reared in a laboratory environment are colonized with the engineered symbiont by adding it to their diet. Finally, knockdown of gene expression is validated using qRT-PCR on RNA isolated from bee tissues. This procedure can be completed from start to finish in one month.

Development of the protocol

The discovery of a core community of gut bacteria in honey bees[11–13] laid the groundwork for the development of FUGUES. This community is remarkably conserved and stable throughout a worker honey bee's adult life. One species of particular importance, *Snodgrassella alvi,* closely associates with the gut lining[13] and induces host immune responses that regulate microbiome composition[14]. Shortly after the discovery of these core gut bacteria, Kwong *et al.* axenically cultured some of these species, including *S. alvi*[15]. They additionally showed that *S. alvi* could be re-administered to honey bees to achieve colonization with laboratory isolates[16].

We subsequently developed the bee gut microbiome toolkit (BTK) for genetically modifying multiple species of bee gut bacteria[17]. The key components of the BTK include promoters that function in diverse bacteria and a broad-host-range plasmid that is compatible with using conjugation to deliver DNA to bacteria lacking established transformation protocols. These universal plasmid components also allow the BTK to be used for genetically modifying other bacteria, including insect symbionts such as *Serratia symbiotica* from aphids[18].

Recently, we used these approaches for culturing and genetically manipulating symbionts to engineer *S. alvi* to alter bee gene expression and protect bees from pathogens[10]. We reasoned that *S. alvi* would be a suitable platform for affecting host biology because (1) *S. alvi* colonizes bees exceptionally well, even from a starting inoculum of only a few cells[10], and (2) *S. alvi* lives in the bee gut in close association with host tissues as a biofilm lining the ileum[11,13]. These characteristics may facilitate exchange of bioactive molecules between *S. alvi* and its bee host.

Using *S. alvi* as a platform for symbiont-mediated RNAi, we demonstrated honey bee FUGUES by knocking down expression of an insulin receptor, which increased the weights of adult worker bees and their sensitivity to sucrose in a feeding assay[10]. Knockdown of the target gene was measured 5 days after colonization and persisted through at least 10 days after colonization. While longer times were not tested, we expect symbiont-mediated RNAi gene knockdown will last throughout the entire lifetimes of colonized bees in the laboratory. We also showed that symbiont expression of dsRNA sequences targeting other organisms could be used to improve bee survival following exposure to a virus and to kill mites that parasitize bees[10].

Applications of the method

Symbiont-mediated RNAi in honey bees can be used for functional genomics and pathogen protection. By knocking down genes and discovering their functions using FUGUES, researchers may also uncover new ways of regulating gene expression to benefit honey bee health or improve pollination efficacy. These methods are intended for laboratory experiments and must be used only for research under approved institutional biosafety and biocontainment protocols. Further testing will be needed to establish whether genetically modified *S. alvi* can be used safely in normal apiculture where colonized bees would mix with other bees in the environment.

The FUGUES approach described here can potentially be applied to study other bee species, particularly those with *S. alvi* or closely related *Snodgrassella* species in their core microbiomes. *Snodgrassella* are abundant in the microbiomes of two additional *Apis* species (*A. andreniformis*

and *A. cerana*) as well as many or all bumble bee species (*Bombus* spp.)[19–22]. Additionally, certain stingless bees contain *Snodgrassella*, including *Partamona helleri*, *Trigona* spp. (*T. hyalinata* and *T. spinipes*), and *Tetragonula* spp. (*T. davenporti*, *T. pagdeni*, and possibly *T. carbonaria*)[20,23]. Both bumble bees and stingless bees are ecologically important natural pollinators, and the former are also used by commercial pollination services[23,24]. Therefore, these groups of insects are attractive targets for symbiont-mediated RNAi using our platform. Because *Snodgrassella* tend to be better at colonizing the bee species with which they have co-evolved[16,20], different species and strains of this symbiont may be more effective for *Bombus* and stingless bee applications. Engineering other *Snodgrassella* to express dsRNA using the approach detailed here is expected to induce a similar RNAi response in other bees.

Beyond bees, symbiont-mediated RNAi has been described in three other insect species – kissing bugs (*Rhodnius prolixus*), western flower thrips (*Frankliniella occidentalis*), and diamondback moths (*Plutella xylostella*) – indicating wider potential applications[9,25–27]. FUGUES leverages a toolkit for assembling broad-host-range plasmids with an RSF1010 origin of replication that is compatible with both Gram-positive and Gram-negative bacteria[17,28]. Therefore, the dsRNA-expression plasmids described here are expected to function in many bacterial species, including other symbionts. More broadly, our hope is that this protocol can serve as a resource for groups interested in establishing symbiont-mediated RNAi and FUGUES in other insect and animal species for which suitable bacterial symbionts can be identified.

Comparison with other methods

The function of a honey bee gene can potentially be studied by using genetic engineering techniques that disrupt its sequence or alter its expression[29]. Transposons have been used to insert expression cassettes into the honey bee genome in two studies[30,31]. Unfortunately, this technique is difficult to employ because it requires laborious injections of embryos and achieves only a low mutagenesis efficiency. More recently, CRISPR/Cas genome editing has been used to more efficiently generate mutant drones or mutant females by a number of different labs[29,32–39]. However, CRISPR/Cas-based methods also require technically demanding injections. A further complication with many genetic engineering approaches is that rearing bee colonies with genetically modified queens is resource-intensive and requires specialized containment facilities.

Though they are not without their own challenges, approaches that use RNAi to knock down gene expression in individual bees are currently the mostly widely used methods for studying bee gene function[40–80]. RNAi is an effective and reliable method in diverse invertebrate species, including *C. elegans* and various insect model systems, but RNAi has had varying degrees of success in honey bees[29,43,81]. The degree of gene knockdown by RNAi can be variable and depend on multiple factors, including target-specific effects (strength of target gene expression and tissue being targeted), dsRNA sequence design, dsRNA dosage, and method of dsRNA administration[29,43,81]. To date, the majority of studies implementing RNAi in bees have involved either dsRNA injection or feeding[81].

Injecting purified dsRNA is commonly used to knock down genes in *A. mellifera*[35,81]. This approach requires expression and purification of dsRNA from an *in vitro* system or *E. coli*[81,82]. It can be expensive and challenging to produce sufficient quantities of high-quality dsRNA.

dsRNA injection leads to variable knockdown depending on the gene and tissue. Results have ranged from a strong >70-75% decrease in mRNA levels for *Vg* and *amGPdh* to a weaker ~30-40% decrease in mRNA levels for *AmOA1* or the hypopharyngeal amylase gene, for example[29,43,46,52,53,57,61,64]. Despite its wide usage, dsRNA injection has a few major drawbacks[81,83]. First, it requires specialized equipment and a high level of technical skill. Second, the manual process of injecting individual bees presents a major bottleneck that limits throughput. Third, gene knockdown by dsRNA injection is necessarily transient, as the injected dsRNA has a finite half-life. Finally, the injection process causes physical trauma to the bee, potentially initiating stress responses and complicating the interpretation of downstream assays.

Feeding purified dsRNA has also been used to induce RNAi in *A. mellifera*[81]. Like injection, this approach requires expression and purification of dsRNA. In contrast to injection, large numbers of bees and even whole hives can be fed dsRNA, as demonstrated in studies that suppress bee pathogens such as Israeli acute paralysis virus and *Varroa* mites[76,84,85]. dsRNA feeding has also been used to knock down bee gene expression, though – as with injection – the effectiveness varies depending on the gene and tissue[29,43,61,66,74,75,78–81]. Another downside of this approach is that it requires continuous addition of dsRNA to the bees' diet to sustain gene knockdown.

This protocol describes a novel method of dsRNA delivery. The FUGUES approach of using symbionts to deliver dsRNA has key advantages compared to conventional methods of administering dsRNA via feeding or injection. First, FUGUES leads to both sustained and robust gene knockdown because dsRNA is produced *in situ* and is continuously delivered from the symbiont to the host. Second, to our knowledge, FUGUES offers the highest scalability of any gene knockdown approach in bees, since a large batch of bees can be readily inoculated with a small amount of engineered *S. alvi*. Finally, feeding engineered *S. alvi* to bees is much simpler and less invasive than dsRNA injection. In short, FUGUES leverages the power of the symbiont to more readily facilitate gene knockdown in bees compared to existing methods.

Expertise needed to implement the protocol

This protocol assumes that the reader has access to an apiary and experience working with honey bees. We provide instructions that relate to special considerations for obtaining uncolonized bees and administering engineered bacteria to them. However, the details of honey bee husbandry and maintenance are outside the scope of this protocol. All microbiology, cloning, conjugation, and molecular biology experiments can be performed in a standard molecular microbiology laboratory with access to a CO_2 incubator for culturing *S. alvi* and a qPCR machine.

Limitations

Some limitations of the current iteration of honey bee FUGUES should be considered before a researcher begins this protocol. FUGUES is only suitable for use in adult worker bees, as larvae and pupae do not harbor the gut symbiont used to deliver dsRNA. As a result, genes involved in early development at the larval and pupal stages cannot be studied. Because FUGUES constitutively induces the host RNAi response beginning early in an adult bee's life, it may perturb normal host physiology in ways that complicate the study of genes that function at later life stages, when bees transition to different worker roles, for example. Additionally, FUGUES

has not been tested in queen bees, which have different gut microbiomes from workers. We also caution that biosafety and biocontainment protocols approved by an investigator's institution must be put into place before using this procedure, as with any genetically modified organisms. Because honey bee hives are typically maintained outdoors in open environments that do not meet these requirements, in-hive behaviors cannot be tested without access to specialized containment facilities. Furthermore, because an antibiotic is used to maintain the engineered plasmid/symbiont, the bee microbiome is not in its natural state during a FUGUES experiment. FUGUES has been demonstrated to facilitate gene knockdown in a range of tissues (including the gut, abdomen, and head)[10]. However, because the mechanisms of dsRNA transfer to and processing in bee cells have not yet been determined, it is not known whether decreased gene expression in tissues distal to the gut is due to direct gene knockdown or secondary, indirect effects. Some of these limitations could be addressed in the future by engineering bee symbiont species that colonize larvae, integrating dsRNA constructs into the bacterial chromosome, or using inducible promoters that allow for temporal control of dsRNA expression.

Experimental design

Here, we provide more detailed overviews of each of the four main phases of this honey bee FUGUES protocol. In addition to providing specific information regarding materials and methods, we pay special attention to controls and decision points where a researcher will need to adjust parameters to suit their study.

Controls

The most important FUGUES control is to test bees colonized with *S. alvi* expressing a non-targeting negative control dsRNA at the same time as bees colonized with *S. alvi* expressing a dsRNA targeting the gene of interest. Comparing on-target results to this negative control allows one to distinguish gene expression and phenotypic effects resulting from knockdown of the targeted gene from nonspecific immune responses that can result from exposing bees to any exogenous dsRNA[10]. We provide a negative control plasmid that expresses a non-targeting dsRNA with a sequence from a green fluorescent protein (*gfp*) gene. We also describe the construction of and provide preassembled dsRNA expression plasmids for two positive controls that can be used to validate the expected results for overall protocol and help with troubleshooting intermediate steps. These control plasmids target the honey bee *insulin receptor 1* (*inR1*) and *defensin-1* (*def1*) genes for knockdown. Finally, we provide a plasmid that does not express any dsRNA as an additional, optional negative control for researchers interested in differentiating the effects of no dsRNA expression from off-target dsRNA expression.

Construct dsRNA expression vector (Steps 1-16)

The first phase of FUGUES is to construct a dsRNA expression vector targeting a honey bee gene for knockdown. This protocol can be adapted to any bee gene with a sufficient length that a dsRNA can be designed that matches part of it and there is enough remaining sequence for designing qRT-PCR primers for monitoring knockdown. While deciding which gene should be chosen for knockdown will ultimately depend on the question a researcher is trying to answer,

we provide a few general design guidelines here. Remember that FUGUES will only affect gene expression during the adult phase of a bee's life because this is when it is colonized with *S. alvi*. The functions of genes at earlier developmental phases cannot be studied using this approach. Also, be sure to check for paralogs of the gene of interest. If these exist, consider designing multiple constructs to test the effects of knocking down each of the related genes separately.

Once a gene has been selected, a piece of it is cloned into a dsRNA expression plasmid. To do this, a knockdown region from the coding sequence of the gene of interest, typically 400 bp or longer, is chosen. If the gene of interest has multiple transcript isoforms, an alignment should be performed to generate a consensus sequence so that all of them or a specific subset can be targeted for knockdown, as desired. Then, PCR is used to amplify the knockdown region from either genomic DNA or cDNA and append BsaI cut sites to each end. Following PCR, amplicons are purified and cloned into a dsRNA expression vector. The assembled plasmid is transformed into *E. coli* cells and plated. Plasmid is isolated from non-fluorescent colonies which do not express GFP, and proper insertion of the dsRNA construct into the plasmid is validated by sequencing. Primers for constructing the positive control plasmids that express dsRNAs targeting either *inR1* or *def1* are provided as a resource for troubleshooting these steps (see Step 2).

At this stage, qPCR primers should also be designed to amplify a ~100 bp region of the coding sequence. These will be used later in the protocol for validating gene knockdown. qPCR primers must amplify a region outside of the dsRNA knockdown region, to prevent them from detecting dsRNA produced by *S. alvi*. They should be designed so that they capture all transcript isoforms of interest. We provide sequences for *inR1* and *def1* qPCR primers that can be used to validate that gene expression is being knocked down using the positive controls (see Step 3).

Engineer bee symbiont to express dsRNA (Steps 17-31)

After the dsRNA expression vector has been made, it is transferred to the bee symbiont *S. alvi*. To do this, the dsRNA expression vector must first be transformed into an auxotrophic, conjugation-competent *E. coli* strain, such as MFD*pir* (which is auxotrophic for diaminopimelic acid, DAP). Other auxotrophic conjugation-competent *E. coli* strains could also be used, such as ST18, a 5-aminolevulinic acid auxotroph[86]. Electrocompetent stocks of MFD*pir* are made and electroporated with the dsRNA expression vector. Transformation reactions are plated on media containing spectinomycin and DAP following recovery. The following day, MFDpir *E. coli* colonies transformed with the dsRNA expression vector are picked and saved as glycerol stocks.

To initiate conjugation, MFD*pir* cells with the dsRNA expression vector and *S. alvi* cells are grown, washed, and combined. Then, this mixture is plated on media containing DAP. The next day, cells are scraped, washed, and plated on media containing spectinomycin (but no DAP), to select for *S. alvi* cells that have acquired the plasmid and to select against MFD*pir* cells. Transconjugant *S. alvi* colonies are passaged onto a second plate containing spectinomycin. After 2-3 days of growth, glycerol stocks are made from a final scrape of these plates.

The plasmids pNR and pDS-GFP serve as negative controls for gene knockdown. pNR is an empty plasmid that does not express any dsRNA. pDS-GFP expresses a dsRNA that does not match any bee sequence. The pDS-Def1 and pDS-InR1 plasmids can used as positive controls

for demonstrating knockdown of targeted genes via FUGUES. Plasmids being used as controls must be transferred into *S. alvi* if this is the first time a researcher is using this protocol. Any of these plasmids can also be used as positive controls for the electroporation and conjugation steps.

Colonize bees with engineered symbiont (Steps 32-42)

Once *S. alvi* is engineered to express dsRNA, it is ready to use to colonize bees. The engineered symbiont is struck out onto an agar plate. For each experiment, engineered *S. alvi* strains are used from at least two groups: the experimental group (*S. alvi* containing a plasmid targeting a gene of interest) and the non-targeting negative control group (*S. alvi* containing pDS-GFP). Including an additional positive control group (*S. alvi* containing pDS-Def1 or pDS-InR1) is optional but highly recommended for new users of this protocol. An additional negative control group (*S. alvi* containing pNR) can also be added, but this is only necessary if a researcher is interested in investigating whether symbiont dsRNA production, irrespective of the target sequence, has an effect on a gene or phenotype of interest. Twenty-four hours prior to bee colonization, engineered *S. alvi* is passaged onto a fresh agar plate. Serially passaging *S. alvi* is not recommended, as lab-adapted symbionts may be less effective at colonizing bees.

Bees should now be obtained for colonization through one of two methods: (1) allowing young adult bees to naturally emerge on their own from the frame or (2) pulling pupae and allowing bees to finish development in plastic brood chambers. Bees that emerge on their own are easier to obtain in large numbers but may also have environmental bacteria present in their guts. Bees obtained by pulling pupae are nearly germ-free, but this procedure is more laborious and requires more technical expertise. The timing of these methods differs, so it is important to decide which method will be used early in the process of planning an experiment. Both methods are described in Step 35, with the method in which bees emerge on their own presented as the default.

When bees have been obtained for colonization, the agar plate containing engineered *S. alvi* is scraped and washed. The *S. alvi* suspension is diluted and mixed with a bee feeding solution. Bees (20-30 per condition) are then dunked in this solution to initiate grooming behaviors that result in symbiont uptake and colonization. Colonized bees are transferred to cup cages and, after a brief fast, are given a sucrose solution supplemented with spectinomycin. Bees are maintained in the lab, replacing their pollen and sucrose diet every three days.

Validate gene knockdown (Steps 43-75)

After one week, bee samples are collected and gene knockdown is validated using qPCR on bee cDNA. For each condition and timepoint, approximately ten bees are removed from cup cages and frozen. Depending on where the gene of interest is expressed, RNA for assessing knockdown can be isolated from entire bees or dissected heads, abdomens, guts, or other body parts. Extracted RNA is then copied into cDNA through reverse transcription. qPCR primers for the gene of interest and reference gene are then tested for adequate efficiency. Subsequently, qPCR is performed on bee cDNA derived from the experimental group (gene of interest knocked down) and control groups (Positive control: *inR1* or *def1* knockdown; Negative control: *gfp* mock knockdown), using primers for the gene of interest and multiple reference genes (such as *rps18* and *gapdh*)[87,88]. Ct values determined by qPCR are used to calculate ΔΔCt, which is used to

determine the relative expression of the gene of interest (compared to the reference gene) for the experimental and control groups[89,90]. Gene expression data are visualized with graphing software and statistical analyses are performed to determine whether a significant reduction in expression of the target gene has been achieved.

Depending on the desired outcome, bees can be assessed for phenotypes of interest or gene expression changes resulting from the dsRNA treatment as early as 3-5 days after colonization. Multiple time points can be analyzed to assess the persistence of gene knockdown over time. We have tested that knockdown persists through least 10 days after colonization[10] and do not have any evidence that it disappears or is attenuated at any point later in the lives of bees.

Materials

Biological Materials

NEB 5-alpha Competent *E. coli*, High Efficiency (NEB, #C2987H) (Store at −80 °C)
Snodgrassella alvi strain wkB2 (ATCC #BAA-2449) (Store at −80 °C)
! Critical Use wkB2 or an equivalent strain known to colonize honey bees.
MFD*pir* or similar auxotrophic *E. coli* conjugation donor strain (e.g., ST18, DSM 22074) (Store at −80 °C)
Hives containing *Apis mellifera*
! CAUTION When handling live adult bees, wear proper protective gear to prevent stings and be prepared in case of life-threatening allergic responses to stings.

Reagents

Construct dsRNA expression vector

Plasmid pBTK800 (RRID:Addgene_179209) or plasmids pBTK403, pBTK301, pYTK002, pYTK072, pBTK150, pBTK151 (RRID:Addgene_110599, RRID:Addgene_110593, RRID:Addgene_65109, RRID:Addgene_65179, RRID:Addgene_183126, RRID:Addgene_183127) (Store at −20 °C)
DNeasy Blood & Tissue Kit (QIAGEN, #69504)
Quick-RNA Tissue/Insect Kit (Zymo, #R2030)
1st strand cDNA Synthesis Kit (Takara, #6110A) (Store at −20 °C)
Q5 Hot Start High-fidelity 2X Master Mix (NEB, #M0494S) (Store at −20 °C)
QIAquick PCR purification kit (QIAGEN, #28104)
Golden Gate Assembly Kit, BsaI-HFv2 (NEB, #E1601S) (Store at −20 °C)
Quick Plasmid Miniprep Kit (PureLink, #K210010)
LB broth and agar plates (Store plates at 4 °C) (BD Difco #244610)
SOC medium (Invitrogen #15544034)
Spectinomycin (Spectinomycin dihydrochloride pentahydrate) (CAS #22189-32-8)
Chloramphenicol (CAS #56-75-7)
Glycerol (CAS #56-81-5)
Molecular grade water (DNase-free and RNase-free) (CAS #7732-18-5)
Primers designed for cloning dsRNA region for gene of interest (IDT) (see Step 2)

Engineer bee symbiont to express dsRNA

Negative control plasmid pDS-GFP (RRID:Addgene_183129) (Store at −20 °C)
Negative control plasmid pNR (optional) (RRID:Addgene_183131) (Store at −20 °C)
Positive control plasmids pDS-Def1 and/or pDS-InR1 (optional)
(RRID:Addgene_183125 and RRID:Addgene_183130) (Store at −20 °C)
2,6-Diaminopimelic acid (DAP) (CAS #583-93-7)
! CAUTION This reagent is an eye and skin irritant. Handle with gloves and protect exposed skin with a lab coat.
5-aminolevulinic acid (if using ST18 instead of MFD) (Sigma-Aldrich, CAS #5451-09-2)
Columbia Blood Agar Base (Hardy-Diagnostics, #C5451)
Sterile Sheep's Blood (Lampire, 7239001-1LTR) (Store at 4 °C)
! CAUTION If contaminated, this reagent may harbor pathogens. Handle with gloves.
Phosphate-Buffered Saline (PBS) (Gibco #10010023)

Colonize bees with engineered symbiont

Sucrose, (CAS #57-50-1)
Pollen (Prairie River Honey Farm, Bulk Fresh Bee Pollen Pure Raw Natural Nebraska Bee Pollen 6 lbs; Pollen shipped by user to Sadex Corporation for irradiation) (Store at −20 °C)

Validate gene knockdown

Quick-RNA Tissue/Insect Kit (Zymo, #R2030)
1st strand cDNA Synthesis Kit (Takara, #6110A) (Store at −20 °C)
DNase I Set (Zymo, #E1010) (Store at −20 °C)
RNA Clean and Concentrator-25 (Zymo, #R1017)
OneTaq 2X Master Mix with Standard Buffer (NEB, #M0482S) (Store at −20 °C)
SYBR Green PCR Master Mix (Thermo-Fisher, #4309155) (Store at −20 °C)
qPCR primers designed for gene of interest (IDT) (see Step 3) (Store at −20 °C)
qPCR primers for reference gene (IDT) (see Step 3) (Store at −20 °C)

Equipment

Construct dsRNA expression vector

Computer with DNA analysis software (Benchling, Geneious, or other)
Shaking incubator (New Brunswick I24Benchtop Incubator Shaker)
Benchtop centrifuge with rotor for 1.5 mL tubes (Eppendorf Centrifuge 5430)
Thermocycler (Eppendorf Mastercycler Nexus Gradient)
Agarose gel electrophoresis system (Galileo Bioscience Minigel System)
Mini-Beadbeater-96 cell disruptor (BioSpec Products, #1001)
Molecular biology grade consumables (Pipette tips, pipette, tubes, racks)
Cryovials (Thermo Scientific, #37518)
Nanodrop (Thermo Scientific NanoDrop Lite Spectrophotometer)
Benchtop spectrophotometer that can measure OD_{600} (Eppendorf BioPhotometer, #6131)
Disposable cuvettes (Thermo Fisher, #14955127)

Engineer bee symbiont to express dsRNA

Electroporator (Bio-Rad Gene Pulser Xcell Electroporation System)
Electroporation cuvettes (1 mm) (Thermo Fisher, #FB101)
CO_2 incubator (Panasonic CO_2 Incubator, #KM-CC17RU1A)
Microbiological loops, 1 µL (Biologix, #65-001)

Colonize bees with engineered symbiont

Standard honey bee husbandry equipment (hives, bee suit, smoker, smoker fuel, hive tool)
Custom built frame cage (constructed as previously described[91])
Cup cage materials (see EQUIPMENT SETUP)
-Plastic cups (Solo, #TP10D)
-Petri dish lids (Corning, #BP93B-102)
-Filter paper (Thermo Fisher, #09-795C)
-96 well plate for pollen trough (Greiner, #655101)
-Tape (Fisherbrand, #15901R)
-10 ml tubes for cup cage feeders (Sarstedt, #62.9924.283)
Environmental chamber with humidity control for rearing bees in lab (Percival Incubator, #I36NL)
50 mL conical tubes (Corning, #430829)
Vacuum filter bottles (Nalgene, #567-0020)
Ice bucket

Validate gene knockdown

qPCR machine (Eppendorf Mastercycler ep realplex Real-time PCR System)
Computer for qPCR machine
96 or 384 well qPCR plates (Bio-Rad HSL9645)
Optical quality plate covers (Excel scientific, #09-795C)

Reagent setup

Sucrose solution (50% [w/w] sucrose:water) for honey bees

Mix 8 lb sucrose with 1 gallon hot tap water, then filter sterilize. Store at room temperature (25 °C) for up to a year.

LB media and agar plates

Prepare media according to manufacturer's instructions. Prepare selective LB agar plates by adding spectinomycin to a final concentration of 60 µg/mL. Prepare selective LB agar plates by adding chloramphenicol to a final concentration of 20 µg/mL. Pour agar into sterile petri plates and let them cool and solidify overnight. Depending on humidity, plates should be dried before use by leaving uncovered in a sterile environment such as a laminar flow hood for 30 minutes prior to use. Store at 4 °C for up to four weeks. Store at 4 °C for up to four weeks.

DAP stock solution for supplementing media

Add 342.4 mg of DAP to 30 mL of H_2O to create a 60 mM stock solution. Shake at 37 °C for ~30 min to fully dissolve, then filter sterilize. This is a 200× stock. Use at a final concentration of 0.3 mM in media. Store aliquots frozen at −20 °C for up to a year.

Columbia Blood Agar Plates
Combine 44 g Columbia media into 950 mL sterile H_2O with a stir bar. Autoclave at 121 °C for ≥ 20 minutes. Remove media from autoclave and let cool to < 60 °C. Add 50 mL sterile sheep's blood. For selective plates, add spectinomycin to a final concentration of 30 μg/mL to molten agar with blood. Pour agar into sterile petri plates and let them cool and solidify overnight. Depending on humidity, plates should be dried before use by leaving uncovered in a sterile environment such as a laminar flow hood for 30 minutes prior to use. Store at 4 °C for up to four weeks.

EQUIPMENT SETUP

Honey bee cup cages
Poke multiple small airholes into the sides of a plastic cup and one hole that snugly fits a 10 ml feeding tube into bottom of the cup. In a fume hood, use a flame heated paring knife to cut grooves into a 96 well plate so that it is possible to snap the plate into sections of 2×3 wells, to act as pollen troughs. Place filter paper onto the lid of a petri dish. Place one pollen trough on the petri dish lid and fill it with irradiated pollen. Place cup (with holes) upside down, covering the pollen trough, onto the petri dish lid and tape to secure (Fig. 4c). Bees (20-30 per cup) can be added through the hole that will hold the feeding tube. When feeding bees, place a 10 ml tube that has small holes poked into the tapered end with a needle into the hole in the bottom of the cup and fill it with sucrose solution. When not using a feeding tube, place a piece of tape over hole on top of the cup to prevent bees from escaping. Feeding tubes can be reused if they are sterilized by soaking in a 10% (v/v) bleach solution for at least 10 minutes, rinsed thoroughly in water, and allowed to air dry between experiments. Prepare cup cages prior to bee colonization (Step 39), so that they are ready to use before the researcher is actively handling bees.

Procedure

Construct dsRNA expression vector ● Timing 9 d

Identify bee gene for knockdown

1. Choose a bee gene for knockdown. This choice will depend on the researcher's specific interests, but the target gene should be associated with a known or hypothesized phenotype. Once a gene of interest has been chosen, to begin the steps needed for cloning the dsRNA expression vector (Fig. 2a), either download the latest honey bee genome assembly (Amel_HAv3.1, GCF_003254395.2 as of 08/20/2022) from NCBI and find the target gene locus using genome viewing software, such as Geneious (paid license required, https://www.geneious.com) or locate and show the sequence of the target gene locus using the online NCBI Genome Data Viewer (available for free use, https://www.ncbi.nlm.nih.gov/genome/gdv/browser/genome/?id=GCF_003254395.2).

▲**CRITICAL STEP** Potential sources in the literature for inspiration for identifying genes of interest may include RNAi studies in *A. mellifera* utilizing fed or injected dsRNA, RNAi studies in other organisms, or studies assessing gene expression profiles in *A. mellifera*.

Design dsRNA expression plasmid

2. Design primers that amplify a portion of the gene of interest's coding sequence (the "knockdown target"; see Fig. 2b). Forward and reverse primers should be 18-24 nucleotides in length, with annealing temperatures compatible both with each other and the polymerase to be used for amplification. Common practice is for the knockdown target to be at least 400 bp long, if possible. This length is not a hard requirement, and shorter targets may be successful in silencing shorter genes. Increasing the target length, up to the length of the full gene, may improve target knockdown, but also increase the chances of inducing off-target effects. Targets can be designed so that they either bridge an exon-intron junction or are entirely contained within an exon. This choice determines how the targets are PCR amplified in Step 7. For simplicity, we recommend targeting sequences contained within an exon, if possible. The chosen knockdown sequence should not contain long homopolymer repeats or other low-complexity sequences. The E-RNA*i* web tool (https://www.dkfz.de/signaling/e-rnai3/idseq.php) can be used to search the *A. mellifera* genome for potential off-target matches. If off-target matches are predicted, we advise picking a new region for knockdown if the length of the gene permits. We show our design for the knockdown of two bee genes: *insulin receptor 1* (*inR1*), which has been previously reported[10], and *defensin-1* (*def1*), which we describe for the first time here, in Fig. 2b. Primer sequences for constructing these dsRNA expression plasmids are provided in Table 1.

3. Design one or two qPCR primer pairs for assessing gene knockdown. These primers will not be used until Step 51, but designing them beforehand at this early step is important to ensure the amplified products of these primer pairs do not overlap with the targeted region of the gene that will be expressed from the symbiont as dsRNA.
▲ **CRITICAL STEP** Use the IDT qPCR PrimerQuest Tool (https://www.idtdna.com/pages/tools/primerquest) with default settings. To ensure good efficiency, these primers should amplify a small target (~100 bp) and have comparable GC contents. We recommend using a target annealing temperature of 60 °C to match the qPCR primers previously designed for the reference gene *rps18*[10]. At this point, one should also order qPCR primers for reference bee genes such as *rps18* and *gapdh*[87,88]. Primer sequences for using qPCR to monitor knockdown of the two example bee genes that can be used as positive controls (*inR1* and *def1*), as well as for reference genes (*rps18* and *gadph*), are shown in Table 2.

4. Append the following nucleotide sequences to the 5′ end of the target amplification primers designed in Step 2. These sequences introduce Golden Gate compatible cut sites (bolded nucleotides below) and overhangs (underlined nucleotides below) for BsaI assembly of the PCR product into the final dsRNA expression vector[17].

Forward sequence (5′-3′): GCATCGTCTCATC**GGTCTC**ATATG
Reverse sequence (5′-3′): ATGCCGTCTCA**GGTCTC**AGGAT

5. Order DNA oligonucleotides for assembly and qPCR from IDT or other DNA synthesis service. There is no requirement for special purification.

Amplify dsRNA knockdown sequence

6. Obtain *A. mellifera* DNA. Depending on the target design, the PCR template will be either *A. mellifera* genomic DNA or cDNA. If the desired cDNA target sequence within a gene does not bridge any introns, the researcher may use genomic DNA to amplify the target, which can be isolated as described in option A. If the target design bridges an intron sequence, however, the researcher should use cDNA prepared from *A. mellifera* RNA as described in option B, as the template to exclude the intron. Alternatively, the researcher may order the entire knockdown region as a gBlock (IDT) or similar dsDNA gene fragment.

A. Isolate genomic DNA from honey bees using the QIAGEN DNeasy Blood & Tissue Kit

(i) Isolate genomic DNA from honey bees using the QIAGEN DNeasy Blood & Tissue Kit according to manufacturer's instructions. DNA can be isolated from one or more whole bees. Eluted DNA will be used as template for the PCR reaction in Step 7.

B. Prepare cDNA from *A. mellifera* RNA.

(i) Isolate RNA from adult *A. mellifera* using the Zymo Quick-RNA Tissue/Insect Kit according to manufacturer's instructions. RNA can be isolated from one or more bees, and can be prepared from pulled guts, whole abdomens, bee heads, or whole bees. Use an appropriate body region that expresses your gene transcript at a high level. Eluted RNA will be used as template for reverse transcription into cDNA.

(ii) Prepare cDNA using Takara 1st strand cDNA Synthesis Kit according to manufacturer's instructions. Use at least 100ng RNA as template, or more for poorly expressed genes. Either random hexamer or polyT primers can be used as primers for the cDNA reaction. Synthesized cDNA will be used as template for the PCR reaction in Step 7.

7. Amplify the knockdown target sequence using the constructed primers and appropriate template DNA. Use NEB Q5 Hot Start High-fidelity 2X Master Mix and follow manufacturer's instructions on reaction setup and thermocycling steps.

8. Verify amplification of a DNA product of the expected size for the knockdown target using agarose gel electrophoresis suitable to verify the size of your knockdown target (Fig. 2c). Generally, a 1% agarose gel loaded with 5-10 uL of PCR product and run for 30 minutes at 100 volts is sufficient.
? TROUBLESHOOTING

9. Purify the desired DNA product using the QIAGEN QIAquick PCR purification kit, following manufacturer's instructions.

10. Quantify the DNA concentration of purified PCR product using a Nanodrop, following manufacturer's instructions. A DNA concentration ≥ 5 ng / uL is sufficient to proceed with assembly.

Assemble dsRNA expression plasmid via Golden Gate Assembly

11. Prepare plasmids for dsRNA expression vector assembly. This reaction can be performed in one of two ways: (option A) a "two-part" assembly reaction with plasmid pBTK800 and PCR product from Step 10; recommended) or (option B) a "seven-part" assembly reaction using plasmids pBTK403, pBTK301, pYTK002, pYTK072, pBTK150, pBTK151, and PCR product from Step 10. pBTK800 is a fluorescent dropout vector described for the first time in this protocol for the simpler and more efficient two-part assembly reaction. The two-part assembly is recommended for a typical user. The seven-part assembly reaction was used previously[10]. It allows components of the plasmid, such as the forward and reverse promoters or the antibiotic resistance marker, to be switched out with other BTK-compatible parts[17]. It may be useful for researchers who want more flexibility for designing customized dsRNA expression vectors.

A) "Two-part" assembly reaction

(i). Prepare purified pBTK800 plasmid. Streak from frozen stock onto an LB plate supplemented with spectinomycin. Incubate at 37 °C overnight.
▲CRITICAL STEP All colonies should be visibly fluorescent from expression of GFP.

(ii) Pick a single fluorescent colony into 3-5 mL liquid LB with spectinomycin and grow overnight with shaking in a 37 °C incubator.

(iii). Isolate plasmid from a stationary phase culture using the PureLink Quick Plasmid Miniprep Kit. pBTK800 has an RSF1010 origin of replication and is a medium copy number plasmid in *E. coli* (~10-20 copies per cell).
▲CRITICAL STEP Follow kit protocol recommendations for low copy plasmids and use ~5 mL of overnight culture for the minipreps to purify a sufficiently concentrated sample of this plasmid (≥ 20 ng/μL).

(iv). Quantify plasmid concentration. Use a Nanodrop to quantify the DNA concentration of purified plasmid following the manufacturer's instructions. A DNA concentration ≥ 10 ng / μL is sufficient to proceed with assembly.
▲CRITICAL STEP DNA quantification methods based on binding of fluorescent dyes to dsDNA, such as those used by a Qubit Fluorometer, will give incorrect values for the concentration of purified RSF1010 plasmid samples because they contain significant amounts of single-stranded plasmid DNA.

B) "Seven-part" assembly reaction

(i). Prepare purified pBTK403, pBTK301, pYTK002, pYTK072, pBTK150, pBTK151. For each plasmid, streak from frozen stocks onto LB plates supplemented with either chloramphenicol (pBTK301, pYTK002, pYTK072, pBTK150, pBTK151) or spectinomycin (pBTK403).

(ii). Pick a colony of each strain into 3-5 mL liquid LB supplemented with the appropriate antibiotic. Cells with pBTK403 express RFP and all others are non-fluorescent. Grow these cultures overnight with shaking in a 37 °C incubator.

(iii). Isolate plasmids from stationary phase cultures as described in Step 11Aiii. pBTK403 has an RSF1010 origin of replication and should be purified in the same way as pBTK800. All other plasmids are high copy number. For these, sufficient yields can be purified from 1.5 mL of culture using normal procedures within the plasmid purification kit.

(iv). Quantify plasmid concentrations as described in Step 11Aiv. A DNA concentration ≥ 10 ng / µL for each plasmid is sufficient to proceed with assembly.

12. Assemble the dsRNA expression vector. Use the NEB Golden Gate Assembly Kit (BsaI-HFv2) mix according to the manufacturer's instructions to set up a BsaI Golden Gate Assembly reaction to assemble plasmid(s) from Step 11 and PCR product from Step 10. Thermocycle the reaction as recommended by NEB.

Transform dsRNA expression plasmid into E. coli *cloning strain*

13. Transform 1-2 µL of the assembly reaction mixture into NEB 5-alpha Competent *E. coli* (High Efficiency) cells according to manufacturer's instructions. Allow cells to recover by incubating at 37 °C for at least one hour. Then, plate 100 µL directly from this mixture and from one or two 10-fold serial dilutions of the mixture in LB or saline separately onto LB plates supplemented with spectinomycin. Incubate plates overnight at 37 °C.
▲ CRITICAL STEP Colonies containing properly assembled plasmid will be non-fluorescent, while colonies that are fluorescent contain the original, unmodified plasmid (Fig. 2d). Agar plates can be placed over a blue light box to visualize fluorescent colonies. If using Step 11A, colonies with incorrectly assembled plasmid will exhibit green fluorescence. If using Step 11B, colonies with incorrectly assembled plasmid will exhibit red fluorescence.
? TROUBLESHOOTING

14. Pick at least three non-fluorescent colonies into 5 mL LB with spectinomycin. Incubate at 37 °C in a shaking incubator.

15. Isolate assembled dsRNA expression plasmids from 5 mL culture, as described in Step 11A(iii).

16. Verify that each assembled dsRNA expression plasmid contains the desired insert using whole plasmid sequencing or targeted Sanger sequencing that covers the cloning junctions between different fragments. Save frozen stocks of transformants with verified inserts by adding glycerol to a final 15% (v/v) concentration to *E. coli* cultures.
▲ CRITICAL STEP Sanger sequencing is not straightforward, given that the insert is flanked on both sides by junctions to identical promoters with sequences that are reverse complements of one another. Use of primers that bind to the vector backbone and are oriented to sequence into the insert may yield unreliable reads under standard sequencing conditions. Three approaches have proven more reliable. The first is to perform Sanger sequencing using primers that bind to the vector backbone under "difficult template conditions" (sometimes referred to as "short hairpin [shRNA] conditions"). Another approach is to perform Sanger sequencing using primers that bind within the sequence cloned from *A. mellifera* for expression as dsRNA, making sure to include primers that will result in sequencing across both junctions to verify insertion. The final

alternative approach is to perform PCR reactions that span both junctions on a plasmid sample, and then Sanger sequence these PCR products.
■ **PAUSE POINT** Freezer stocks can be stored for >1 year at −80 °C.

Engineer bee symbiont to express dsRNA ● Timing 9 d

Transform dsRNA expression vector into E. coli *conjugation strain*

17. Several steps are needed to transfer the dsRNA expression vector to *S. alvi* (Fig. 3a). Firstly prepare an electrocompetent stock of an auxotrophic *E. coli* donor strain, such as Mu-free donor (MFD*pir*) or ST18, according to a standard electrocompetent cell preparation protocol[92] (Box 1).
▲ **CRITICAL STEP** For the remainder of the protocol, this auxotrophic donor strain will be referred to as MFD*pir*, but ST18 could also be used. ST18, which is available from DSMZ (DSM 22074), is a conjugation-competent *E. coli* strain auxotrophic for 5-aminolevulinic acid[86]. If using ST18, substitute 5-aminolevulinic acid (50 µg/ml)[86] for DAP.

18. Electroporate 1 µL of purified dsRNA expression plasmid (from Step 16) into one aliquot of electrocompetent MFD*pir* cells (see Box 1). Use the standard *E. coli* setting on the Bio-Rad electroporator compatible with 0.1 cm cuvettes (V = 1.8 kV).
▲ **CRITICAL STEP** If this is a researcher's first time performing this protocol, they must also construct a strain of *S. alvi* with the negative control plasmid pDS-GFP. We also recommend that new users follow this protocol with at least one of the plasmids pDS-InR1 and pDS-Def1, which serve as optional positive controls for demonstrating knockdown of a targeted bee gene. If a researcher wants to test whether a non-targeting dsRNA has an effect on expression of the target gene, they should also construct a strain of *S. alvi* with the optional pNR negative control plasmid, which does not express any dsRNA, for comparison to pDS-GFP results. *E. coli* cells containing each of these control plasmids can be obtained from Addgene (see REAGENTS). Steps 13-31 should be followed to move control plasmids into *S. alvi* in parallel with the target gene plasmids.

19. Allow electroporated MFD*pir* to recover in SOC medium supplemented with DAP for 1 hour at 37 °C in a shaking incubator.

20. Plate 100 µL of two to three 10-fold serial dilutions of transformed MFD*pir* onto separate LB plates supplemented with DAP and spectinomycin. Plate a non-transformed control. Incubate at 37 °C overnight and look for colonies the next day (Fig. 3b).
? TROUBLESHOOTING

21. Pick three colonies into liquid LB supplemented with spectinomycin and DAP. As a control, inoculate 3 test tubes of LB supplemented with spectinomycin (but without DAP) using the same colonies. Incubate overnight at 37 °C with shaking.
▲**CRITICAL STEP** MFD*pir* donors should only grow in the presence of DAP (Fig. 3b). This is an important control to ensure that the transformed MFD*pir* cells are not contaminated prior to the conjugation.

22. Make frozen stocks of verified MFD*pir* transformants by adding sterile glycerol to 15% (v/v). It is typically not necessary to sequence the plasmid again at this stage.

■ **PAUSE POINT** Freezer stocks can be stored for >1 year at −80 °C.

Transfer dsRNA expression plasmid into S. alvi

23. Prepare for conjugation into *S. alvi* wkB2. Streak from frozen *S. alvi* stock onto a Columbia Blood agar plate and incubate for 48 hours at 35 °C under 5% CO_2 (Fig. 3c). Grow the donor strain (MFD*pir* with dsRNA expression plasmid) by adding 1-2 µL of the freezer stock to 3-5 mL of LB supplemented with spectinomycin and DAP and incubating overnight at 37 °C with shaking. Begin growth of the donor strain one day later than wkB2 so that both are ready at the same time for the next step.

24. Scrape a loopful of wkB2 (Fig. 3c, Supplementary Video 1) into PBS in a 1.5 mL tube. Transfer 1 mL of MFD*pir* donor culture into a 1.5 mL tube.

25. Centrifuge both tubes at 3824 × g for 5 minutes at room temperature. Discard supernatants and resuspend pellet from each tube in 1 mL PBS.
▲**CRITICAL STEP** Resuspending the *S. alvi* pellet will require repeated pipetting, as *S. alvi* forms a dense and sticky aggregation during growth.

26. Measure the OD_{600} of each tube using a benchtop spectrophotometer.

27. Combine wkB2 and MFD*pir* at a ratio of 9:1 by OD_{600} to a total volume of 100 µL and plate on Columbia Blood agar plates supplemented with DAP. As a control, spot wkB2 by itself (no donor) (Fig. 3d) and MFD*pir* by itself (no recipient). Incubate all plates at least overnight and up to 24 hours at 35 °C under 5% CO_2, allowing both MFD*pir* and wkB2 to replicate (Fig. 3d)

28. After overnight growth, use a sterile loop to scrape bacterial growth into 1 mL PBS in a 1.5 mL centrifuge tube. Use a pipette to resuspend the mixture and then centrifuge at 3824 × g for 5 minutes at room temperature. Discard supernatant and add 1 mL PBS. Pipette to resuspend the cell pellet. Centrifuge again at 3824 × g for 5 minutes, discard supernatant, and resuspend in 1 mL PBS as before.
▲**CRITICAL STEP** This wash step is necessary for removing residual DAP that could lead to continued MFD*pir* growth.

29. Plate dilutions (50 µL, 10 µL, 1 µL) onto Columbia Blood agar plates supplemented with spectinomycin, but without DAP. Incubate plates at 35 °C under 5% CO_2 for 48 hours.

30. Successful transconjugant colonies should be visible after 48–72 hours (Fig. 3e). Streak 1–3 colonies onto fresh Columbia Blood agar plates supplemented with spectinomycin. Incubate for 48 hours at 35 °C under 5% CO_2.
? TROUBLESHOOTING

31. Scrape transconjugant wkB2 growth directly into a cryovial with 15% (v/v) glycerol. Mix well by pipetting to break up adhesive clumps of *S. alvi* and store at −80 °C. These stocks will be used for inoculating honey bees. These transconjugants can be confirmed to be pure *S. alvi* cultures by performing 16S rRNA sequencing to ensure no unexpected contaminants have been introduced during the conjugation process.
■ **PAUSE POINT** Freezer stocks can be stored for >1 year at −80 °C.

Colonize bees with engineered symbiont ● Timing 11–32 d

Revive S. alvi *to prepare for inoculating bees*

32. Several coordinated steps must be carried out to colonize bees with the engineered symbiont (Fig. 4a). Three days prior to the planned inoculation day, streak out colonies from frozen stocks of *S. alvi* + dsRNA expression vector (constructed in Step 31) and the control strain *S. alvi* + pDS-GFP (non-targeting negative control). All strains should be struck on Columbia Blood agar plates supplemented with spectinomycin. Incubate strains for 48 hours at 35 °C under 5% CO_2. Inspect for normal growth or contaminants.
▲**CRITICAL STEP** All knockdown trials must include a symbiont strain expressing a non-targeting dsRNA (*S. alvi* + pDS-GFP). This negative control is used as a baseline for comparing knockdown of the gene of interest (*S. alvi* + dsRNA expression vector). If you suspect that expression of your gene is affected by a general RNAi response[44], you should also include a symbiont strain with the empty expression plasmid that does not express any dsRNA (*S. alvi* + pNR) as an additional negative control for comparison to the other treatments.

33. After 48 hours (one day before planned inoculations), restreak a loopful of bacterial of each strain onto a Columbia Blood agar plate supplemented with spectinomycin. This second passage will be used for inoculation after 24 hours of incubation in Step 35.

Collect bees from apiary

34. Collect bees according to one of two methods: option A; allow adult bees to emerge from the frame or option B; pull pupae and allow bees to develop in plastic brood chambers. Option A can be performed on the same day as Step 33, whereas option B should be initiated 3-4 days prior.
! CAUTION Severe allergic reactions to bee venom can be life threatening. Ensure you follow your institution's policies for working with venomous animals. When handling live bees in the following steps, wear proper protective gear to prevent stings. Prepare beforehand to have another individual present who is ready to take appropriate medical responses immediately if a researcher has a known allergy or displays signs of an allergic response.

A. Allow bees to emerge

(i). Inspect the beehives for a suitable brood frame by gently removing the caps of brood cells with the hive tool and noting the age of pupae.
▲**CRITICAL STEP** The frame should contain a brood patch with pupae that are ready to emerge, have dark eyes, and sclerotized heads (20-21 days old). A frame that contains a small number of bees in a brood patch already chewing through their brood caps works well.

(ii). Move the frame into the laboratory using an enclosed frame carrier.
(iii). Incubate the frame in a growth chamber at 35 °C and 80% relative humidity overnight to allow for adult bees to emerge.

B. Pull pupae and allow bees to develop in brood chambers

(i). Inspect the beehives for pupae 3-4 days earlier than for emerged bees.
▲CRITICAL STEP Look for a brood frame containing late-stage pupae with pigmented eyes but no movement (17 -19 days old).
(ii). Move the frame into the laboratory using an enclosed frame carrier.
(iii). Once in the lab, use sterile forceps to carefully remove the cell caps from a patch of brood (Fig. 4b).
(iv). Use sterile forceps to remove individual pupae (Fig. 4b) and place each into a sterile dish provisioned with sterile pollen and 1:1 sucrose:water solution.
▲CRITICAL STEP Flame sterilize forceps periodically to ensure no cross-contamination.
(v). Place the dishes with pupae into a growth chamber at 35 °C and 80% relative humidity, and allow pupae to finish development over the next 3-5 days.
▲CRITICAL STEP Once pupae have transitioned into adult emergers and are moving around the dish, they are ready for inoculation.

Inoculate bees with S. alvi

35. *S. alvi* strains are ready for inoculation into bees after 24 hours of growth on agar plates. On the day of inoculation, scrape several loopfuls of bacteria from each agar plate from Step 33 into 1 mL PBS. Use a 1 mL pipette to resuspend the bacteria (to break down biofilm and facilitate dilutions). Centrifuge in a tabletop centrifuge at 3824 × g for 5 minutes at room temperature. Remove PBS and replace with 1 mL fresh PBS. Pipette up and down to resuspend bacteria.
! CAUTION The following Steps (36 – 44) must be conducted under contained laboratory conditions. Follow biosafety protocols approved by your institution for housing, handling, and disposing of bees colonized with bacteria that have been genetically engineered with recombinant DNA.

36. Prepare a 1:10 dilution of the bacterial suspension in PBS and read OD_{600} using a spectrophotometer. Dilute this mixture with PBS to an OD_{600} of 0.1. Add 200 µL diluted bacterial suspension to 800 µL of a filter-sterilized 1:1 sucrose:water sterilized solution and mix well by vortexing. The 1 mL of solution prepared in this way is suitable for inoculating 20-30 bees. The precise OD_{600} used to inoculate bees is flexible, as honey bees inoculated with a wide range of *S. alvi* concentrations reliably colonize to the same carrying capacity[10].

37. Using forceps or a gentle finger grip, sort bees from Step 34 into groups of 20-30 and place each group into a sterile 50 mL conical tubes. Newly emerged adult bees (< 24 hours post emergence) can neither sting nor fly, so this sorting can be done without anesthetizing bees. If used, older bees should be chilled briefly at 4 °C before sorting to slow their movement.

▲CRITICAL STEP The number of bees inoculated at this step with each *S. alvi* strain containing a different plasmid should be sufficient such that 8-12 bees will survive through Step 44 when bee samples are saved for subsequent RNA extraction. More bees may need to be

inoculated and analyzed if RNAi knockdown of the target gene decreases bee survival or to detect small changes in gene expression if knockdown of the target gene is weak.

38. Add 1 mL of inoculation solution (prepared in Step 36 above) to each conical tube with bees, close the tube, and gently shake for 10-15 seconds (Fig. 4c). This will coat the bees in the sugary inoculation solution. Allow 2-3 minutes for the bees to begin cleaning each other off. Repeat Steps 37-38 until all experimental groups have been colonized.

39. Transfer each group of 20-30 inoculated bees into a cup cage prepared with sterile pollen (Fig. 4c) and incubate at 35 °C and 80% relative humidity. Allow 30 minutes for bees to entirely clean each other off. The top of the cup cage can be covered with a small petri dish or tape.

40. After 30 minutes, introduce one feeder containing sucrose solution supplemented with 60 µg/mL spectinomycin to each cage.
▲CRITICAL STEP Place the feeder on the pollen trough to catch excess liquid. This feeder should be replaced every 72 hours with fresh sucrose solution and antibiotic. Ensure the feeder tube is neither clogged nor draining too quickly. The feeding solution should be pre-warmed to 35 °C in the incubator to prevent it from excessively leaking when added to cup cages.

Maintain bees and (optionally) validate colonization

41. Maintain bees in an incubator at 35 °C and 80% relative humidity for 5-21 days. The timing will depend on when knockdown of the gene is expected to have an effect on a bee phenotype of interest, which may need to be empirically determined by a researcher.
▲CRITICAL STEP Check daily for any deceased bees and remove them from cup cages. *S. alvi* should fully colonize the bee gut 3-5 days after inoculation. Bees that have been inoculated with the engineered symbionts will generally survive for at least 2-3 weeks in the lab.

42. (Optional) The first time you perform this protocol we recommend validating that colonization of bees with your engineered *S. alvi* was successful. To do so, first incubate bees for 5 days after inoculating them with the symbiont. Then, dissect guts from inoculated bees (see Step 45), homogenize the tissue in a 1.5 ml tube (resuspend in 500 µL PBS and grind with a pestle), plate 100 µL of each of six 10-fold serial dilutions in PBS onto Columbia Blood agar plates containing spectinomycin, incubate at 35 °C, 5% CO_2 for 2-3 days[10], and record CFUs. We typically observe that *S. alvi* numbers gradually climb and then reach a stable level of ~5 × 10^7 CFU per bee by 5 days after colonization.
? TROUBLESHOOTING

Validate gene knockdown ● Timing 2 d

Sample and (optionally) dissect bees

43. The final steps in this protocol test for knockdown of the target gene (Fig. 5a). After a minimum of 5 days, sample bees from the experimental and control groups (positive control: *inR1* knockdown or *def1* knockdown; negative control: *gfp* mock knockdown) by immobilizing them using either CO_2 (option A) or chilling to 4 °C (option B).

(A) Immobilize bees using CO_2

i) Place entire cup cage in a plastic bag and introduce CO_2 until bees stop moving. Bees will be paralyzed within 30 seconds of CO_2 exposure, and remain paralyzed for 1-2 minutes.

(B) Immobilize bees by chilling to 4°C

i) Place entire cup cage in a refrigerated environment and wait until bees stop moving. This may take 30 minutes to 1 hour depending on the number of bees in the cup cage.

44. Working quickly, remove 8-12 immobilized bees from the cup cage and place them in either a 5 mL conical tube for long term storage at −80 °C or directly on a petri dish over ice for immediate processing. Multiple bees from the same treatment can be placed into one 5 mL conical for freezing and several bees can be placed onto a single petri dish for dissection.
■ **PAUSE POINT** Bees can be stored at −80 °C for at least 6 months and then processed for RNA isolation or can be processed immediately.

45. (Optional) If the researcher is specifically interested in assessing gene knockdown in a particular tissue, dissect the appropriate tissue expressing the gene of interest prior to RNA extraction, using option A to obtain the head or abdomen, or option B to obtain the guts.
▲**CRITICAL STEP** Whole guts are recommended for initial tests using the positive and negative controls, as robust gene knockdown is observed in the gut[10]. The type of tissue used will depend on the researcher's experimental aims. If you will only assess gene expression in the whole bee body, then dissection is unnecessary, and you can skip to the next step.

A. Dissecting head or abdomen

(i.). Prepare labelled 1.5 ml tubes with 500 µL PBS. You need one tube per bee.

(ii). Thaw bees on ice before dissection if they were previously frozen at −80 °C.

(iii). Sever the head or abdomen using a clean razor blade for genes expressed in brain tissue or the fat body, respectively.

(iv). Place the dissected tissue into one of the 1.5 mL tubes with 500 µL PBS.

(v). Repeat this procedure for all bee samples using a clean razor blade each time.

B. Dissecting guts

(i). Prepare labelled 1.5 ml tubes with 500 µL PBS. You need one tube per bee.

(ii). Thaw bees on ice before dissection if they were previously frozen at −80 °C.

(iii). Immobilize a bee on a petri dish placed above a bucket of ice by grasping the bee thorax. Using a pair of flat tipped forceps in one hand, grasp the last tergite before the bee stinger with a pair of sterile point-tipped forceps.

(iv). While holding the thorax in place, smoothly pull the point-tipped forceps away from the thorax.

▲ **CRITICAL STEP** As the tip of the abdomen separates from the rest of the abdomen, the entire unpunctured gut should remain attached to the stinger.

(v). Place the whole gut into one of the 1.5 mL tubes with 500 µL PBS.

(vi). Flame or alcohol sterilize forceps and repeat this procedure for all bee samples.

Isolate total RNA from bees and generate cDNA

46. Using either frozen whole bee samples (from Step 44) thawed on ice and placed in a 1.5 ml centrifuge tube, or dissected tissue (Step 45), manually homogenize each sample with a pestle. Then, follow the manufacturer's instructions for RNA extraction using the Zymo Quick-RNA Tissue/Insect Kit. For the initial bead beating step, processing two times for 30 seconds each on a BioSpec Mini-Beadbeater-96 cell disruptor is sufficient.
▲**CRITICAL STEP** Perform the optional DNase I treatment step according to manufacturer's instructions when purifying RNA with this kit.

47. Elute total RNA in 20 µL molecular grade water into RNase-free microcentrifuge tubes.

48. Quantify concentration of RNA using Nanodrop. Sample purity can be assessed from the 260/280 ratio, with a value of 2.0 considered to be pure according to manufacturer's guidelines.

49. Freeze RNA at −80 °C for storage or use immediately for cDNA synthesis.
■ **PAUSE POINT** RNA can be stored at −80 °C for >6 months.

50. Generate cDNA using the Takara 1st strand cDNA Synthesis Kit following the manufacturer's instructions with a 10 µL reaction volume and random hexamer primers.
▲ **CRITICAL STEP** Normalize total input RNA for all samples. We recommend at least 100 ng RNA per sample. Samples with very low concentrations (< 10 ng / µL) of RNA due to improper extraction or RNase contamination should be excluded at this step. All samples from a given experiment that will be compared to each other during qRT-PCR should be processed together to minimize batch effects.

51. Dilute 10 µL of cDNA with 90 µL molecular grade water so that there is a sufficient volume of this template to perform several qRT-PCR reactions on each sample that amplify the relevant reference and test genes.
■ **PAUSE POINT** cDNA can be stored at 4 °C for >2 weeks or −20 °C for >6 months.
▲ **CRITICAL STEP** Repeated freeze-thaw cycles can dramatically reduce the quality of diluted cDNA samples and lead to more variable qPCR results. Once cDNA is generated and diluted it should be stored at 4 °C until all planned qPCR assays are complete.

Test qPCR primer efficiency

52. For each amplicon that will be used to monitor expression of a reference or target gene, add bee cDNA and the corresponding primer set to a 50 µL PCR reaction using OneTaq 2X Master Mix with Standard Buffer according to the manufacturer's instructions. 1–2 µL of diluted cDNA should be enough template. The cDNA can be generated from an untreated bee as described in Step 6 or from an experimental bee as described in Step 50, as long as all relevant transcripts are expressed at levels that provide sufficient template for amplification.

53. Thermocycle the PCR reaction according to the manufacturer's instructions. Choose an annealing temperature that matches that of your designed primers.

54. Confirm amplification of the gene product occurs at the intended size (~100 bp) by performing gel electrophoresis on the PCR product.

55. Clean up the PCR amplicon using the QIAGEN QIAquick PCR purification kit according to the manufacturer's instructions.

56. Elute the purified PCR product in 50 µL molecular grade water. This product serves as the PCR standard for that amplicon. It will be used for testing qPCR primer efficiency here and as an internal control in qPCR reactions on experimental samples in Step 61.

57. Prepare dilutions of the PCR standards for each primer set. Serially dilute 1 µL of purified PCR product with molecular-grade water across eight 10-fold serial dilutions to cover a large dynamic range of amplification. Run qPCR using Bio-Rad 2× SYBR Green Master Mix according to the manufacturer's instructions. Each dilution should be used as template in three qPCR reactions that serve as technical replicates. A single 96-well qPCR plate can be used to run standard curves for up to four sets of primers: 10^{0}-10^{-7} dilutions for each primer pair in triplicate.

58. After completion of the qPCR reaction, generate a standard curve using the Ct values for each dilution and the log of the template DNA concentration.

59. Calculate efficiency using the formula $E = 10^{-(1/\text{slope})} - 1$, inputting the slope of the standard curve generated in Step 58[93,94]. Multiply this value by 100 to get % efficiency.
▲CRITICAL STEP The efficiency of each primer set should be ≥90%. For the ΔΔCt comparison method (described below) to be valid, efficiencies for the reference and test genes should be roughly equivalent[95]. If these values differ substantially, use the Pfaffl method for calculating relative changes in gene expression[95].

Perform qPCR for reference and target genes

60. Make a plate map for the qPCR reactions of interest. Plan to perform qPCR on cDNA samples from at least six bees in each of the experimental and control groups. Each of these cDNA samples is a biological replicate. Analyzing more biological replicates will give greater statistical power for detecting small gene expression differences. Each bee cDNA sample will be used as template in at least two types of qPCR reactions, one with a primer set for a reference gene and one with the primer set for the test gene. If desired, qPCR reactions with primer sets for additional reference genes can be used[87,88]. We recommend performing at least three technical replicates per biological replicate for each primer set to account for qPCR variation. Each technical replicate is a separate reaction in a different well on the qPCR plate.

The results presented here for knockdown of *inR1* (Fig. 5b) and *def1* (Fig. 5c) were obtained using 7-8 biological replicates for each of two sample groups: bees colonized with *S. alvi* + pDS-GFP (the negative control plasmid) and bees colonized with *S. alvi* + pDS-InR1 or pDS-Def1 (the positive control plasmid expressing dsRNA targeting the gene of interest). Each cDNA sample was used as template in three types of qPCR reactions, one with a primer set that amplified a portion of the target gene and two that amplified fragments of either the *rps18* or *gapdh* bee reference genes. All of the qPCR primer sequences are provided in Step 3. Three technical replicates were performed for each combination of a primer set and biological replicate.
▲CRITICAL STEP If possible, plan to set up all samples to be compared on a single qPCR plate to minimize run-to-run variability. For example, a single 96-well plate might focus on one

primer pair. It can accommodate three qPCR reactions (technical replicates) for each of 24 samples (which could be eight biological replicates from each of three sample groups, for example), plus the eight internal control reactions for that primer pair described in the next step in triplicate. For larger experiments, use a 384-well qPCR plate if possible. If this is not possible, ensure each qPCR plate has samples from every experimental group to minimize batch effects.

61. Prepare dilutions of the PCR standards for each primer set. Serially dilute 1 µL of purified PCR product across seven 10-fold serial dilutions. Use pure water for the eighth sample as a negative control.
▲CRITICAL STEP These dilutions and negative control should be included, in triplicate, on every qPCR plate set up using a given primer pair. They serve as an internal control between plates that can be used to verify the expected qPCR amplification results. Primer sets should yield similar efficiency results to those that were obtained in Step 59 each time they are used.

62. Prepare qPCR master mix on ice. For each sample prepare triplicate 10 µL reactions that include:

5 µL Bio-Rad 2× SYBR Green Master Mix
0.5 µL each qPCR primer (10 µM concentration)
3 µL Molecular grade water
1 µL template (diluted cDNA reaction from Step 51)

▲CRITICAL STEP For consistency, we recommended preparing a master mix that contains enough of all of these components (×1.1 for pipetting error) for every sample, except for the unique template. Gently pipette to mix.

63. Dispense 9 µL of master mix into every well of a qPCR plate.
▲CRITICAL STEP The plate should be set up on ice. For consistency and easy setup, we recommend using multichannel pipettes.

64. Dispense 1 µL of each standard prepared in Step 61 in triplicate.

65. Dispense 1 µL of each experimental sample into a well in triplicate.

66. Use a mini plate spinner to collect liquid at the bottom of each well, spinning for roughly 30 seconds at room temperature.

67. Apply an optically clear plastic cover to the qPCR plate.

68. Load the qPCR plate into a pre-heated qPCR machine (Mastercycler ep realplex Real-time PCR System). Perform the thermocycling reaction according to default parameters, according to manufacturer's instructions.

69. At the end of the run, export the raw Ct values (which will be used to determine changes in gene expression, with the ΔΔCt method).

Calculate relative gene expression and run statistical analyses

70. To calculate ΔΔCt manually[89,90], first calculate the mean Ct value for the reference gene and the mean Ct value across all qPCR technical replicates for the test gene for each control sample. Alternatively, use an R package such as `pcr` or `qpcR`[89,96] to assist with computation.

71. Calculate the mean Ct value for the reference gene and the mean Ct value across all qPCR technical replicates for the test gene for each experimental sample.

72. Calculate ΔΔCt for each biological replicate (cDNA sample from a different bee) in the experimental group (*S. alvi* + knockout target dsRNA expression plasmid) and the non-targeting negative control group (*S. alvi* + pDS-GFP plasmid) by subtracting the mean Ct value of all negative control samples from each calculated Ct value.
▲CRITICAL STEP If you included both pDS-GFP and pNR negative control groups, calculate ΔΔCt values by subtracting the mean Ct value of all empty vector negative control samples (*S. alvi* + pNR plasmid) from each calculated Ct value. In this case, the calculated expression levels will be relative to this no-dsRNA negative control instead of the non-targeting negative control.

73. Convert ΔΔCt values to relative expression using the following equation[89]: Relative expression = $2^{-\Delta\Delta Ct}$. Optionally, further $\log_2$ transform the relative expression data.

74. Plot relative expression values (untransformed or $\log_2$-transformed) using graphing software to visualize changes in gene expression (compared to the reference gene) in experimental versus control samples (Fig. 5b-c). Because measured gene expression levels can vary greatly from bee to bee, it is best to plot all individual data points in addition to means and confidence intervals.

75. Analyze the change in gene expression in the experimental group compared to the control group to determine if gene expression knockdown is statistically significant.
▲CRITICAL STEP Perform these tests on ΔΔCt values or $\log_2$-transformed relative gene expression. Check if data points within each group are normally distributed using D'Agostino's K^2 test. If they are, then an unpaired *t*-test can be used to test for a significant difference. If the data are not normally distributed, then the nonparametric Mann-Whitney *U* test can be used.
? TROUBLESHOOTING

Timing

Steps 1–16, construct dsRNA expression vector: 9 d
Steps 17–31, engineer bee symbiont to express dsRNA: 9 d
Steps 32–42, colonize bees with engineered symbiont: 11–32 d
Steps 43–75, validate gene knockdown: 2 d

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 Troubleshooting

Step	Problem	Possible Reason	Solution

8	No PCR product	(1) Reagents accidentally omitted from PCR reaction (2) Incorrectly designed primers (3) Insufficient cDNA template (4) Low quality RNA or cDNA (5) Poor cDNA yield from reverse transcription (6) Low expression of target gene in bee body parts and tissues used for RNA isolation	(1) Include known positive control template (2) Verify proper primer design (3) Increase amount of template DNA in PCR (4) Repeat RNA and cDNA preparation with fresh reagents (5) Increase amount of RNA template in reverse transcription reaction (6) Isolate RNA from a tissue expected to highly express the target gene
13	All colonies after transformation of Golden Gate assembly are fluorescent	(1) Golden Gate assembly enzymes non-functional (2) *E. coli* competent cells have low efficiency (3) Low quality plasmid prep of pBTK800 (or other parts)	(1) Use fresh enzymes and positive control reaction from kit (2) Test transformation efficiency of *E. coli* cells using control plasmids (3) Re-miniprep pBTK800 (or other parts)
20	No transformed colonies of MFD*pir*	(1) Low transformation efficiency of prepared competent cells (2) Low plasmid yield in miniprep of dsRNA expression vector	(1) Test transformation efficiency with control plasmid (2) Culture cells for miniprep at higher volume to increase miniprep yield
30	No *S. alvi* colonies after conjugation	(1) Low conjugation efficiency (2) Contamination and outgrowth of bacterial contaminant during conjugation	(1) Perform control conjugation with original pBTK800 plasmid; also consider increasing the donor:recipient ratio. (2) Use spectinomyin at appropriate concentration and ensure that cells are being washed to remove DAP before final plating

42	No colonization of honey bees with engineered *S. alvi*	(1) *S. alvi* inoculum plate is too old (> 24 hours) (2) Bacterial contaminant (not *S. alvi*) used for colonization	(1) Ensure overnight growth of *S. alvi* used for colonization (2) Perform 16S rRNA sequencing (see Step 31) to verify species of bacteria prior to inoculation
75	No significant knockdown of target gene	(1) Mutation in dsRNA expressing plasmid (2) Target design non-functional (3) qPCR primers not properly amplifying gene (4) Gene not amenable to RNAi	(1) Sequence plasmid to verify promoter region and insert of plasmid in strain used for colonization (2) Design additional dsRNA constructs targeting same gene (3) Design additional qPCR primers (4) Consult literature to determine if gene has been reported to be amenable to RNAi in other organisms

Anticipated results

This honey bee FUGUES protocol yielded 50% to 75% knockdown of gene expression for the positive controls targeting the *inR1* and *def1* genes (Fig. 5b,c). Knockdown of *inR1* by this amount affected bee phenotypes, including altered worker bee feeding behavior and abberant weight gain after emergence[10]. To our knowledge, knockdown of *inR1* or *def1* has not been attempted using other RNAi strategies in honey bees, so it is not possible to directly compare the efficacy of symbiont-mediated RNAi to other techniques. RNAi knockdown of *def1* in bumble bees by injecting dsRNA[97] has been reported to result in a larger decrease in expression than we observed in honey bees using this protocol, but variation in bee physiology, dsRNA design, and other factors could explain this difference.

The efficiency of gene knockdown by RNAi can vary substantially depending on target specific effects (such as native gene expression levels and tissue-dependent expression) and design of the dsRNA[29,43,81]. The qPCR procedure described here is the most direct method to confirm knockdown of gene expression when testing the FUGUES approach on a new gene. Once significant knockdown is established, researchers can move on to study whether there are any effects on organismal phenotypes to provide direct evidence that a gene is involved in a biological process of interest.

Data availability

Source data for figures 2c, 5b, and 5c are provided. Plasmid sequences are available on the Addgene website under the accession numbers provided in the Reagents section. Other data supporting the results of this study are available within the article.

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Author contributions

Conceptualization, S.P.L. and J.E.B.; Methodology, S.P.L., J.E.P., and J.E.B.; Investigation, P.J.L., S.P.L., J.E.P., R.D.H.; Writing, P.J.L., S.P.L., and J.E.B.; Editing, P.J.L., S.P.L., J.E.P., R.D.H., and J.E.B.

Competing interests

S.P.L. and J.E.B. are co-inventors on a patent (US 11,382,989) covering the use of engineered symbionts to improve bee health. J.E.B. is the owner of Evolvomics LLC.

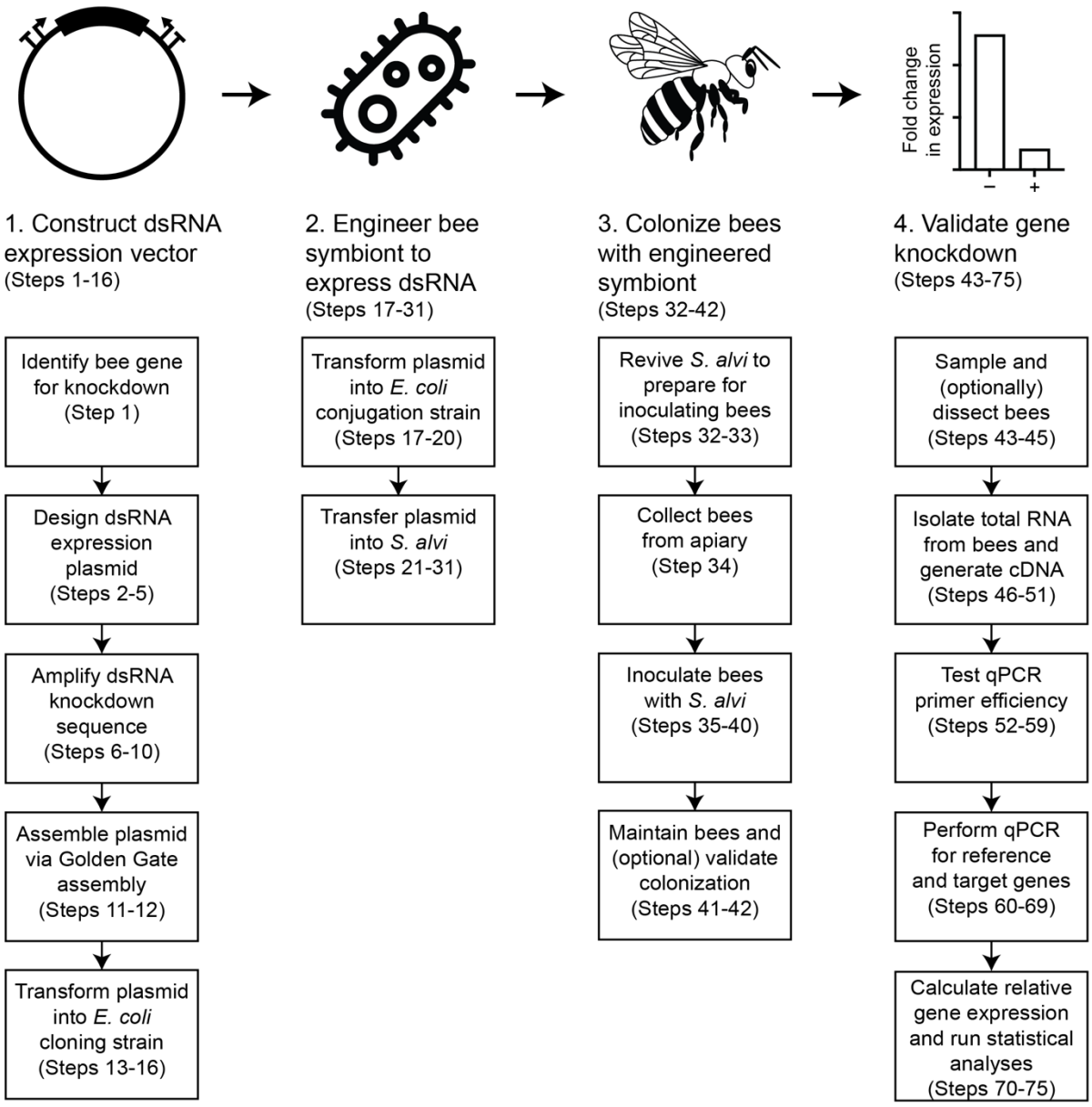

Fig. 1 | Workflow for using symbiont-mediated RNAi to study gene function in bees. Schematic of FUGUES workflow. In phase 1 (cloning) the dsRNA expression vector is designed, constructed using Golden Gate assembly, and transformed into *E. coli*. In phase 2 (transfer to bee symbiont), the dsRNA expression vector is transferred to *S. alvi* via conjugation. In phase 3 (bee inoculation), bees are collected and inoculated with engineered *S. alvi*. In phase 4 (gene knockdown validation), total RNA is collected from bees, reverse transcribed into cDNA, then used as template for qRT-PCR reactions using reference and target gene primers. Fold expression is then calculated to quantify changes in gene expression. The procedure as described takes a total of approximately 31 days, including 18 days to design, build, and validate the plasmid and engineered symbiont strains, 11 days to colonize and then house the honey bees, and 2 days to validate the knockdown. While we describe the process for a single target gene, multiple knockout targets can be designed, built, and tested in parallel.

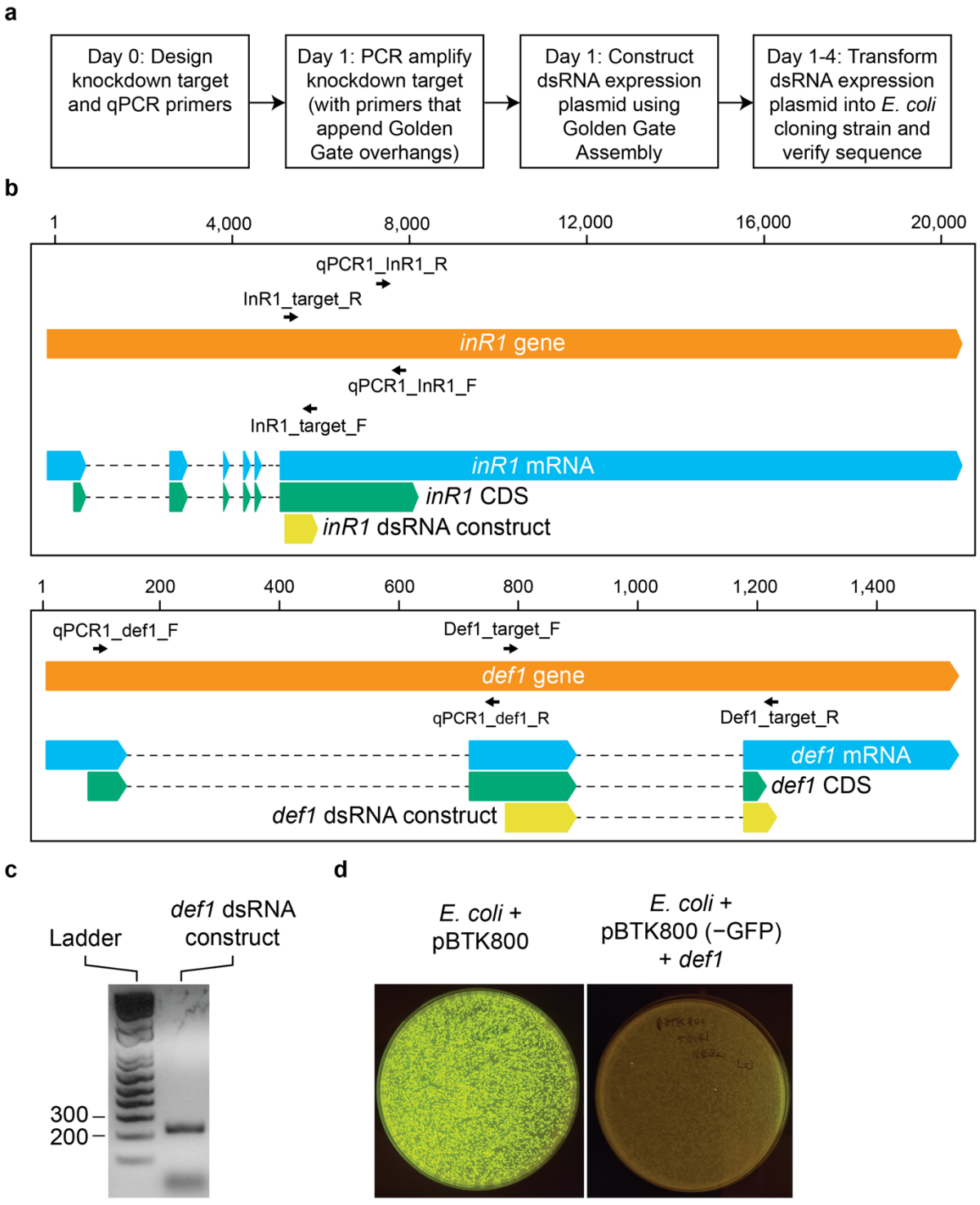
a
Day 0: Design knockdown target and qPCR primers
Day 1: PCR amplify knockdown target (with primers that append Golden Gate overhangs)
Day 1: Construct dsRNA expression plasmid using Golden Gate Assembly
Day 1-4: Transform dsRNA expression plasmid into E. coli cloning strain and verify sequence
b
1
4,000
8,000
12,000
16,000
20,000
qPCR1_InR1_R
InR1_target_R
inR1 gene
qPCR1_InR1_F
InR1_target_F
inR1 mRNA
inR1 CDS
inR1 dsRNA construct
1
200
400
600
800
1,000
1,200
1,400
qPCR1_def1_F
Def1_target_F
def1 gene
qPCR1_def1_R
Def1_target_R
def1 mRNA
def1 CDS
def1 dsRNA construct
c
Ladder
def1 dsRNA construct
300
200
d
E. coli + pBTK800
E. coli + pBTK800 (−GFP) + def1

Fig. 2 | dsRNA expression vector cloning. a, Overview of the dsRNA expression vector cloning workflow. **b**, Maps of example genes chosen for knockdown: *inR1* (top) and *def1* (bottom). Each map displays the architecture of the gene (orange), mRNA (blue), coding sequence (CDS) (green), and dsRNA construct PCR product (yellow). Primer sites for amplifying the knockdown target for *inR1* and *def1* and qPCR primers for both genes are displayed as small black arrows. The horizontal scale (in nucleotides) is shown above each gene map. The *inR1* gene is pictured in the reverse orientation to how it is encoded in the *A. mellifera* chromosome, so its R and F primers appear flipped here. Also note that PCR is performed on honey bee cDNA in these examples, so only the regions present in the mRNA (blue) will be amplified. The *inR1* gene map is an updated version of a previously published figure[10]. **c**, PCR amplification of the *def1* dsRNA target sequence. The amplicon runs at the expected 230 bp size on a 2% agarose gel (200 and 300 bp ladder standards indicated). **d**, *def1* dsRNA expression plasmid assembly. pBTK800 is a GFP dropout vector for constructing dsRNA expression plasmids using Golden Gate assembly. *E. coli* transformed with pBTK800 grow into colonies that are visibly fluorescent under blue light (left). Successful cloning replaces the GFP cassette in pBTK800 with the target sequence. Nearly all *E. coli* colonies are non-fluorescent after transformation with a Golden Gate assembly reaction combining pBTK800 and the *def1* dsRNA target sequence (right). One of these would be picked to verify that it contained the properly assembled dsRNA expression plasmid. An uncropped gel image for panel c is provided as Source data in the Supplementary Information.

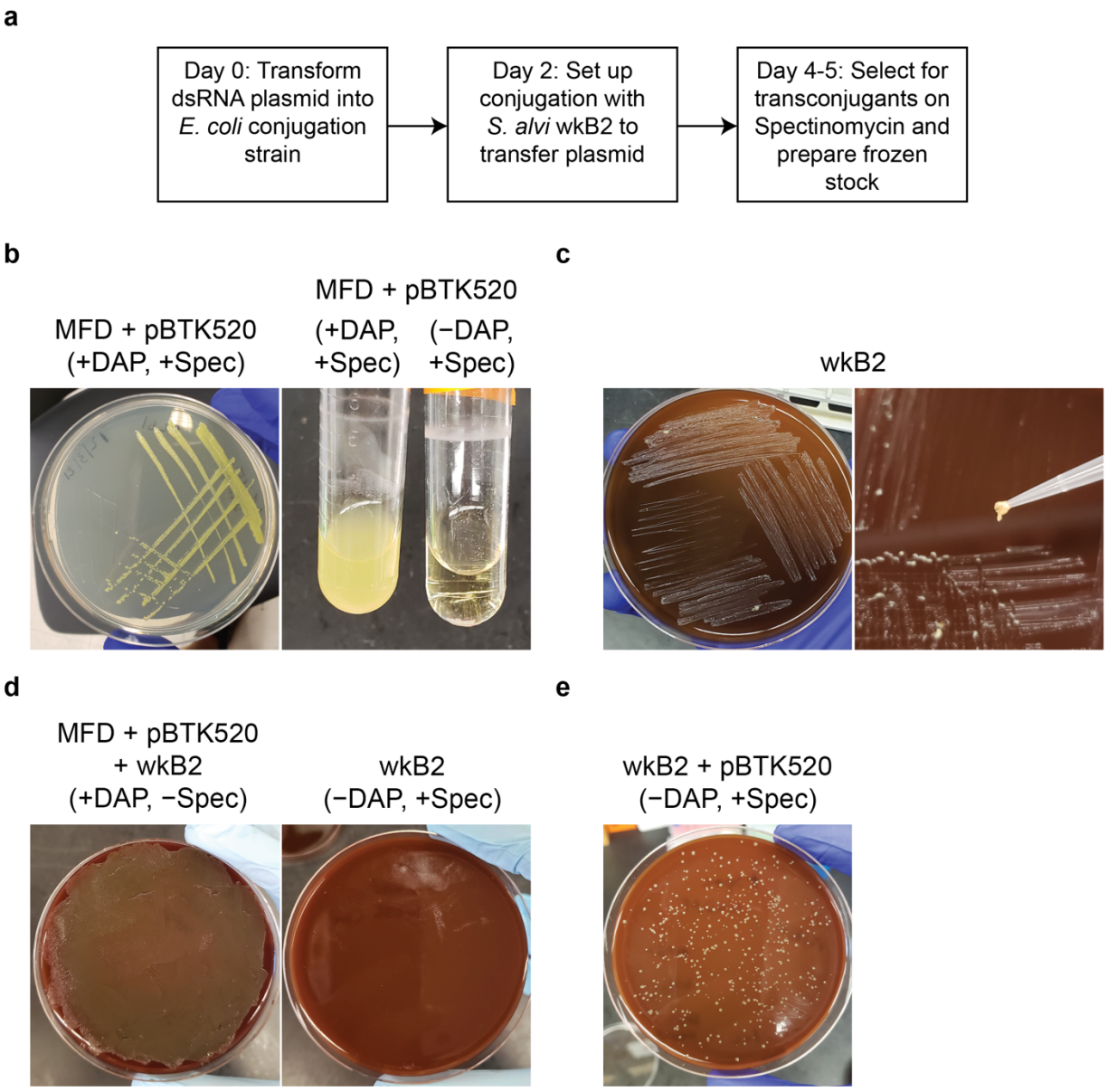

Fig. 3 | Plasmid conjugation into *S. alvi*. **a**, Overview of conjugation workflow. **b**, Conjugation competent *E. coli* donor strain MFD*pir* (MFD) containing a plasmid with spectinomycin resistance, struck out on LB agar +DAP +Spec (left panel). MFD + pBTK520 grown in liquid LB +Spec +/−DAP, showing that MFD is a DAP auxotroph (right panel). The pBTK520 plasmid encodes a constitutively expressed *gfp* that makes *S. alvi* visibly fluorescent[16]. It is used to demonstrate this procedure but is not part of the FUGUES workflow. **c**, Conjugation recipient *S. alvi* wkB2 strain (wkB2), struck out on Columbia Blood agar (left panel). wkB2 colonies are mucoid in texture and yellowish-white in color when scraped (right panel). **d**, Conjugation plate containing 9:1 wkB2 recipient:MFD donor strains grown on +DAP, −Spec plate to facilitate conjugation (left panel). wkB2 control (−DAP, +Spec) does not grow in the presence of Spec (right panel). Both plates are Columbia Blood agar. **e**, Conjugation selection plate (Columbia Blood agar −DAP, +Spec) showing growth of wkB2 + pBTK520 transconjugants.

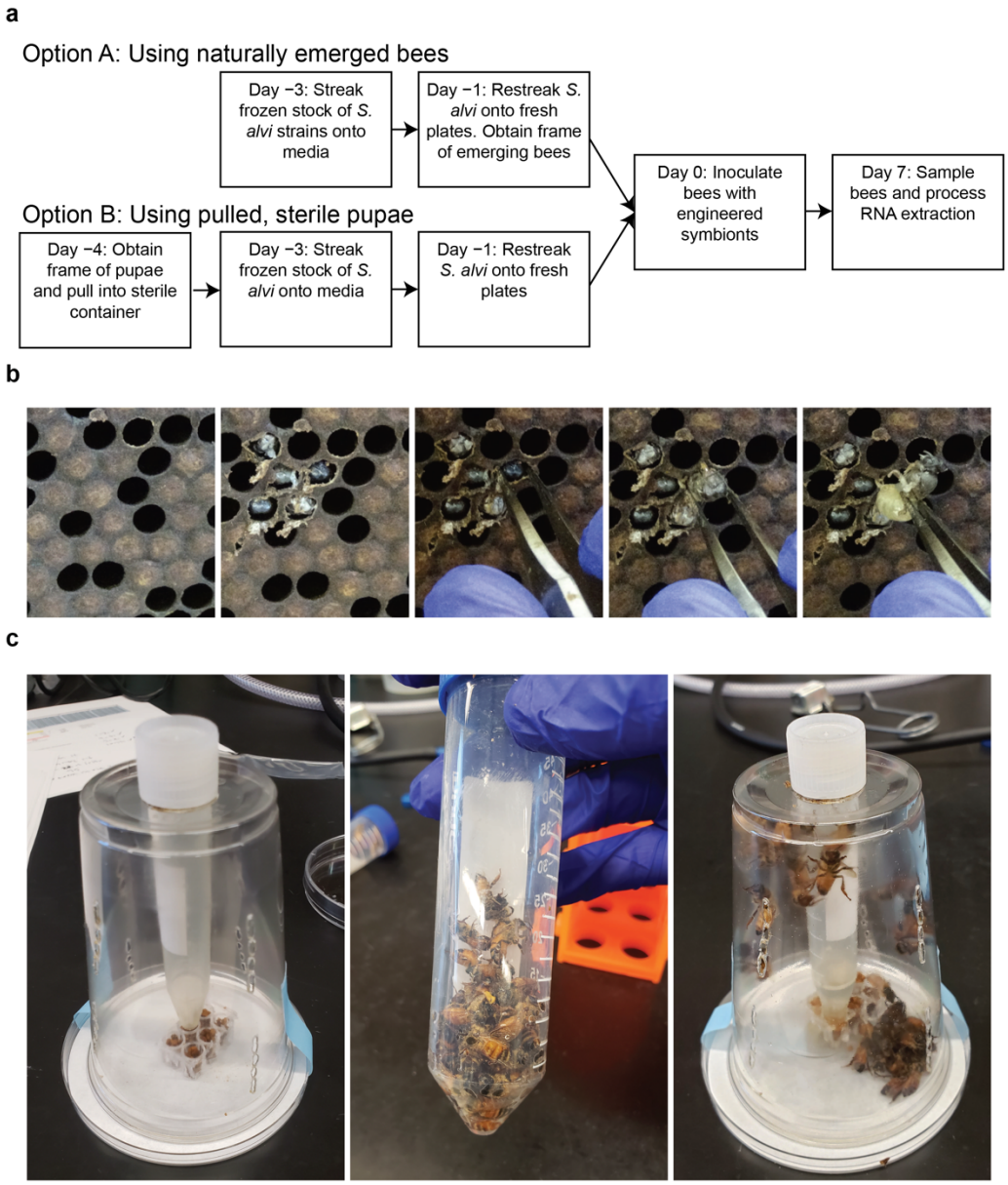

Fig. 4 | Honey bee inoculation with *S. alvi*. **a**, Overview of bee inoculation workflow. **b**, An *A. mellifera* pupa is pulled from a frame (panels move left to right). A frame containing fresh brood is removed from its hive box (left panel) and cells are uncapped (center-left panel) with light scraping. Sterile forceps are carefully inserted into an uncapped cell containing a pupa (center panel), and the pupa is gently pulled (center-right panel) until it is fully removed from its cell (right panel). **c**, *A. mellifera* adults are inoculated with *S. alvi.* Bee cup cages are set up as described, containing a feeding trough with irradiated pollen and a feeding tube for syrup (left panel). Honey bees are inoculated with *S. alvi* by combining newly emerged adults with a syrup solution containing *S. alvi* in a 50 ml conical tube and shaking to mix (middle panel). Inoculated bees are transferred to a cup cage by removing the feeding tube, inverting the 50 ml tube over the hole in the top of the cup, and replacing the feeding tube (right panel).

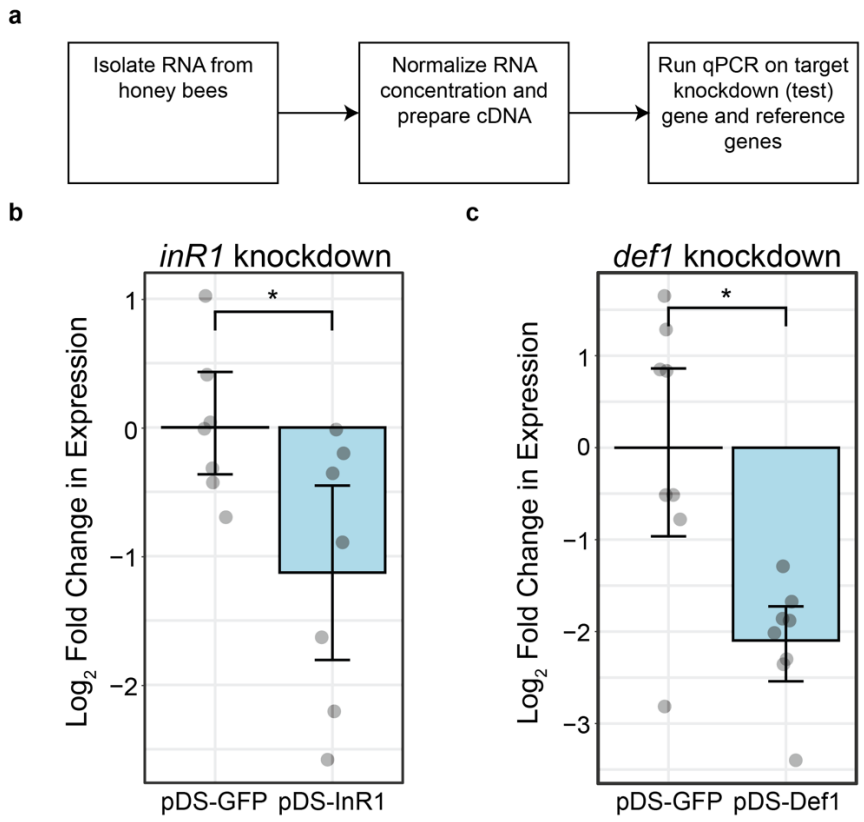

Fig. 5 | Validation of gene knockdown. a, Overview of gene knockdown validation workflow. **b**, Fold expression plot showing decreased *inR1* gene expression in bees treated with the *inR1* knockdown symbiont strain. qPCR was performed on cDNA derived from bee guts that were dissected from bees colonized with *S. alvi* expressing dsRNA sequences matching either *inR1* (pDS-InR1) or *gfp* (pDS-GFP; non-targeting negative control) for 5 days. Expression levels of *inR1* (the test gene) were normalized to *rps18* and *gapdh* (reference genes). Expression of *inR1* was significantly lower in bees treated with the on-target dsRNA versus the non-targeting negative control (Mann-Whitney *U* test, $p = 0.038$). N = 7 for both *inR1* and *gfp*. The *inR1* data is from ref. [10]. **c**, Fold expression plot showing decreased *def1* gene expression in bees treated with the *def1* knockdown symbiont strain. qPCR was performed on cDNA derived from bee abdomens that were dissected from bees colonized with *S. alvi* expressing dsRNA sequences matching either *def1* (pDS-Def1) or *gfp* (pDS-GFP; non-targeting negative control) for 7 days. Expression levels of *def1* (the test gene) were normalized to *rps18* and *gapdh* (reference genes). Expression of *def1* was significantly lower in bees treated with the on-target dsRNA versus the non-targeting negative control (Mann-Whitney *U* test, $p = 0.0070$). N = 8 for both *def1* and *gfp*. In **b** and **c**, the blue boxes show the differences between group means, and error bars are bootstrapped 95% confidence intervals constructed from the measurements in each group. Source data for panels b and c are provided as Supplementary Information.

Table 1. Primers for Assembling dsRNA Expression Plasmids

Primer	Sequence (5' to 3')
inR1 dsRNA Forward	GCATCGTCTCATC**GGTCTC**ATATG*CCAGATTCCTCACCGTTATGTTT ATG*
inR1 dsRNA Reverse	ATGCCGTCTCA**GGTCTC**AGGAT*AATCCGAACATAATGAACGAGTTG AG*
def1 dsRNA Forward	GCATCGTCTCATC**GGTCTC**ATATG*AGAAGAGTAACTTGTGACCTTCT CTC*
def1 dsRNA Reverse	ATGCCGTCTCA**GGTCTC**AGGAT*TGAATCTTCATAATGGCACTTAACC G*

BsaI restriction enzyme recognition sites are bolded. Overhangs resulting from digestion are underlined. Bases that anneal to the cDNA template are italicized.

Table 2. qPCR Primers

Primer	Sequence (5' to 3')
inR1 qPCR Forward	CCCTTCCTCCCTATCTTGTAGTA
inR1 qPCR Reverse	GCGAGGAATTGCATGGTTTC
def1 qPCR Forward	TTTATTGTCGGCCTTCTCTTCA
def1 qPCR Reverse	TCGGCACGTTCTTCATTCT
gapdh qPCR Forward	ACAGACCCGAGTGAATAGATTTG
gapdh qPCR Reverse	CGAACTCAATGGAAGCCCTAA
rps18 qPCR Forward	AGGTGTTGGTCGTCGTTATG
rps18 qPCR Reverse	CATTCTCCAGCACGCTTATCT

Box 1. Electrocompetent cell preparation protocol

Procedure

1. From MFD*pir* frozen stock, streak an LB plate supplemented with DAP and incubate at 37 °C overnight.
2. Pick a single colony into LB media supplemented with DAP. As a control, pick the same colony into LB without DAP. Incubate at 37 °C shaking overnight.
3. Confirm bacterial growth is only in the test tube supplemented with DAP. Dilute the saturated MFD*pir* culture with 10 mL of fresh LB with DAP. Incubate at 37 °C shaking.
4. After 4 hours, begin checking the OD_{600} every 30 minutes until the culture reaches mid-log phase (0.4 – 0.6 OD_{600}).
5. Once the culture has reached mid-log phase, transfer the culture to a 15 ml conical tube, Centrifuge for 5 minutes at 3824 × g at 4 °C, and remove the supernatant.
6. Add 10 ml cold 10% glycerol to the pellet to wash the cells, keeping cells at 4 °C for the remainder of the protocol (keep cells on ice and use a refrigerated centrifuge cooled to 4 °C). Vortex to mix, centrifuge for 3.5 minutes at 3824 × g at 4 °C, and remove the supernatant.
7. Wash in 10% glycerol, as above, three additional times.
8. Add 100 µl of 10% glycerol to the pellet and pipet to resuspend.
9. Dispense electrocompetent cells into 50 µl aliquots, and freeze at −80 °C if not using immediately for electroporation.

Supplementary Information

Supplementary Video 1 | Scraping *Snodgrassella alvi* cells from an agar plate. Movie demonstrating technique used to scrape *S. alvi* colonies from a Columbia Blood agar plate.