BEE HEALTH

Engineered symbionts activate honey bee immunity and limit pathogens

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Honey bees are essential pollinators threatened by colony losses linked to the spread of parasites and pathogens. Here, we report a new approach for manipulating bee gene expression and protecting bee health. We engineered a symbiotic bee gut bacterium, *Snodgrassella alvi*, to induce eukaryotic RNA interference (RNAi) immune responses. We show that engineered *S. alvi* can stably recolonize bees and produce double-stranded RNA to activate RNAi and repress host gene expression, thereby altering bee physiology, behavior, and growth. We used this approach to improve bee survival after a viral challenge, and we show that engineered *S. alvi* can kill parasitic *Varroa* mites by triggering the mite RNAi response. This symbiont-mediated RNAi approach is a tool for studying bee functional genomics and potentially for safeguarding bee health.

Honey bees (*Apis mellifera*) are dominant crop pollinators worldwide and a model organism for studying development, behavior, and learning. Recently, high honey bee colony mortality (*1*), attributed largely to synergistic interactions between parasitic mites (*Varroa destructor*) and RNA viruses (*2*), has become a critical problem for agriculture and the maintenance of natural biodiversity. Despite the importance of honey bees, studies of honey bee biology are limited by bees' unusual social structure and reproductive biology. New genetic tools and methods for deterring pathogens are vital for understanding and protecting honey bees.

Honey bees possess the molecular machinery for RNA interference (RNAi) (*3*), a eukaryotic antiviral immune system in which double-stranded RNA (dsRNA) triggers degradation of other RNAs with similar sequences. RNAi can be induced by feeding or injecting dsRNA, and this has been used to knock down expression of bee genes and to impair replication of RNA viruses, including deformed wing virus (DWV) (*4*–*8*). dsRNA administered to bees is transmitted to their eukaryotic parasites and can induce parasite RNAi responses. This approach has been used to suppress *Varroa* (*9*) and *Nosema* (*10*) by using dsRNAs that silence essential parasite genes. However, use of dsRNA for sustained manipulation of bee gene expression or control of bee pests has proven difficult. Even administration of dsRNA to individual bees yields patchy and transient gene knockdown (*11*), and dsRNA can have off-target effects (*12*–*14*). There are even greater obstacles to using dsRNA to defend entire hives located in the field against pathogens, as dsRNA is expensive to produce and degrades rapidly in the environment.

Here, we describe successful efforts to engineer *Snodgrassella alvi* wkB2, a symbiotic bacterium found in bee guts, to continuously produce dsRNA to manipulate host gene expression and protect bees against pathogens and parasites.

S. alvi is a core member of the conserved gut microbiota of honey bees (*15*). To test whether engineered *S. alvi* robustly colonizes bees, we inoculated newly emerged, antibiotic-treated bees en masse with *S. alvi* transformed with a plasmid expressing green fluorescent protein (GFP) and then monitored bacterial colonization (Fig. 1). Even at a dose of 500 colony-forming units (CFU), engineered *S. alvi* establishes within worker bees, grows to ~5.0×10^7 CFU after 5 days (Fig. 1A), and persists stably throughout the life span of bees reared in the lab (Fig. 1B). Most engineered *S. alvi* cells remained functional throughout our 15-day experiments, although some bees contained cells that lost fluorescence at the final time point (Fig. 1C). We also confirmed that, 11 days after colonization, engineered *S. alvi* was found along the gut wall with the same localization as the wild-type strain (Fig. 1, D to F) (*15*).

To test whether *S. alvi* can deliver dsRNA in situ, we designed a modular platform to assemble plasmids that produce dsRNA from an inverted arrangement of two promoters (fig. S1). First, we assessed whether *S. alvi* produced dsRNA during colonization and whether there was a general bee immune response to symbiont production of dsRNA. We inoculated bees with *S. alvi* wkB2 transformed with either a plasmid that expressed no dsRNA (pNR) or a plasmid that expressed dsRNA corresponding to the GFP coding sequence (pDS-GFP). At 5, 10, and 15 days after inoculation, we sampled and dissected bees to measure RNA levels in different body regions. We detected GFP RNA in the head, gut, and hemolymph of bees colonized with dsRNA-producing bacteria at all sampling times (fig. S2). The presence of GFP RNA in the hemolymphs and heads of bees, where no bacteria reside, suggests that RNA is transported throughout their bodies, as previously reported (*8*). We also detected up-regulation and differential expression of immune pathway genes in the bees colonized with *S. alvi* bearing the pDS-GFP plasmid, and for some genes this up-regulation correlated with the amount of dsRNA produced in the gut (fig. S2). The up-regulated genes included *DDX52* and *DHX33*, which encode RNA helicases previously implicated in the bee immune response to dsRNA (*8*). Other up-regulated genes included *cact1* and *cact2* (in abdomens), which remained up-regulated for the entire 15-day trial; *cact1* and *cact2* were previously shown to be up-regulated after injection of dsRNA, but only for a few hours (*8*). The RNAi components *dicer* and *argonaute* were not consistently up-regulated, but *dicer* expression in abdomens did increase 5 to 10 days after colonization, as reported for *dicer* shortly after dsRNA injection (*8*). Thus, engineered *S. alvi* persistently produces dsRNA in situ, and the bee host responds by activating immune pathway genes.

Next, we tested whether symbiont-produced dsRNA can be used to silence specific host genes. The insulin/insulin-like growth factor signaling pathway controls bee feeding behavior and development, including the transition of worker bees from nurses to foragers (*16*). We built a dsRNA plasmid targeting the insulin receptor *InR1* (pDS-InR1) (Fig. 2A and fig. S3), transformed this plasmid into *S. alvi*, and assayed its effects on bees. Compared with the pDS-GFP off-target control, we saw significantly lower expression of *InR1* over multiple days and in all tested body regions (Fig. 2B). In contrast, previous studies found that direct injections of dsRNA into honey bee brains cause only transient (<1 day) knockdown (*17*). Bees colonized by bacteria harboring the pDS-InR1 plasmid showed increased sensitivity to low concentrations of sucrose (Fig. 2C) and gained more weight over time in each of two independent trials (Fig. 2D and fig S4). *InR1*-suppressing bacteria led to significantly heavier bees at 10 and 15 days after colonization, likely a product of increased feeding behavior. Thus, symbiont-mediated RNAi systemically silences bee genes and can lead to persistent behavioral and physiological changes.

Next, we tested whether symbiont-produced dsRNA can protect bees against a common viral pathogen. We designed three dsRNA-producing plasmids targeting different sections of the DWV genome (pDS-DWV1 to pDS-DWV3) (fig. S5) and then initially assessed whether *S. alvi* with these plasmids could help bees resist DWV infection (fig. S6). We orally inoculated bees with DWV and 48 hours later assessed viral

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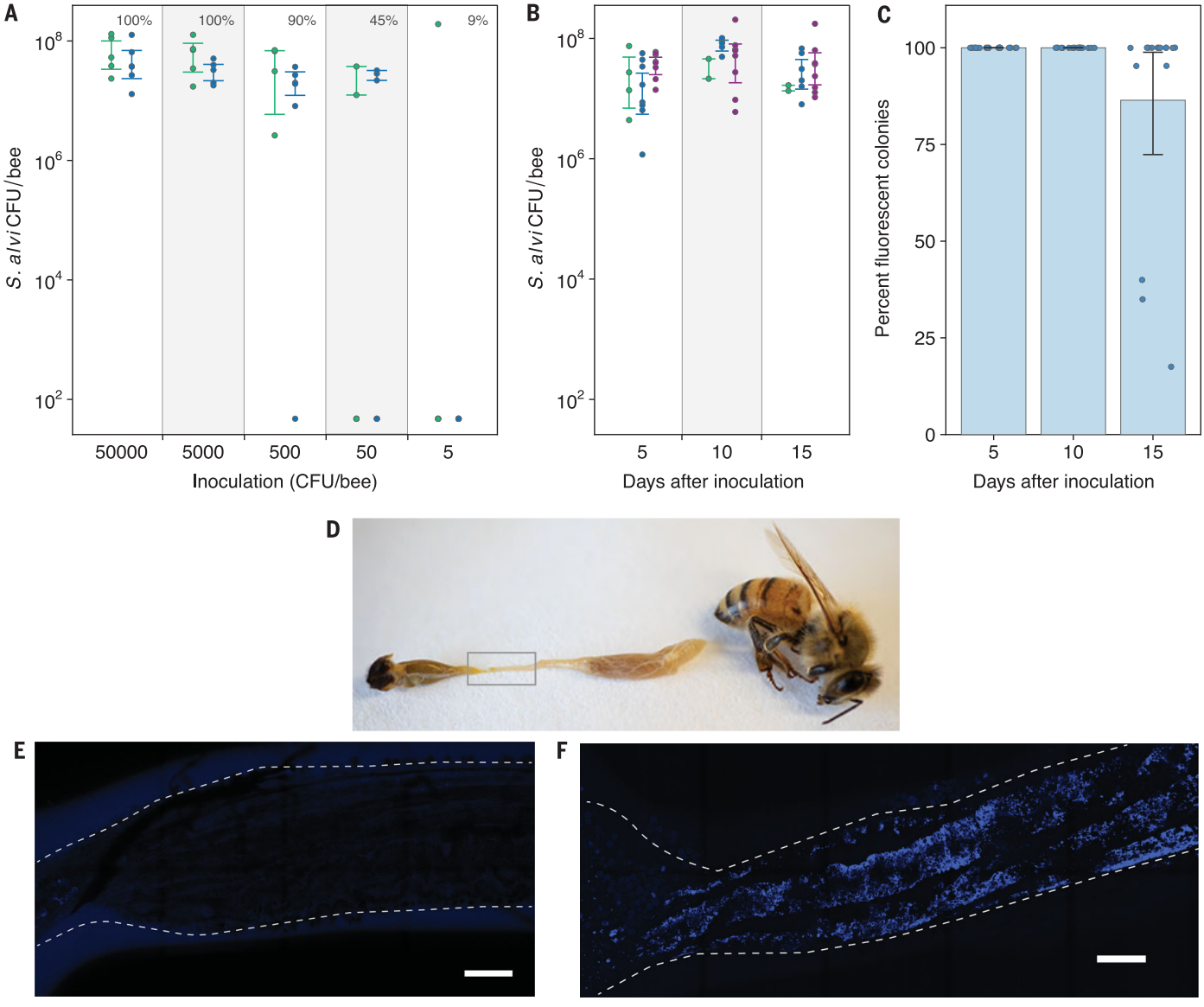

Fig. 1. Engineered *S. alvi* colonizes and functions in bee guts. (**A**) Colonization of newly emerged honey bees by different inoculum sizes. The percentage of bees colonized in each treatment is annotated above the inoculation dose. N = 53 bees from two hives. (**B**) Stability of *S. alvi* colonization over time. N = 48 bees from three hives. Colors in (A) and (B) correspond to different source hives. (**C**) Stability of GFP expression by engineered *S. alvi* over time. (**D**) Photograph of dissected bee. *S. alvi* resides in the ileum (gray box). (**E** and **F**) Ilea of bees 11 days after colonization with nonfluorescent (E) or fluorescent (F) *S. alvi*. E2-Crimson fluorescence from engineered *S. alvi* is blue. Scale bars, 150 μm. Error bars in (A) to (C) are 95% bootstrap confidence intervals.

replication in the hemolymph using a quantitative polymerase chain reaction assay. DWV levels were lower, on average, in bees colonized by *S. alvi* with any dsRNA-producing plasmid, including the off-target pDS-GFP control (figs. S6A and S7). The *dicer* gene was also upregulated in bees inoculated with most dsRNA-producing plasmids after virus exposure (fig. S6B). These results suggest some nonspecific induction of an immune response in bees colonized with *S. alvi* expressing dsRNA. However, only the pDS-DWV2 plasmid significantly increased survival in a separate experiment in which bees were injected with purified virus (fig. S6C).

To validate the latter finding, we performed a larger experiment to assess whether dsRNA-producing bacteria improved survival after DWV injection. This procedure mimics the natural route of DWV transmission via *Varroa* mites feeding on bees (*2*). We injected cohorts of 7-day-old bees with DWV and monitored their survival over 10 days (Fig. 3). After DWV injection, bees with bacteria bearing pNR died rapidly. Likewise, pDS-GFP provided no significant protection. In contrast, pDS-DWV2 significantly improved survival of virus-injected bees. Thus, symbiont-mediated RNAi can protect honey bees from DWV, and it does so in a targeted, sequence-specific manner.

Finally, we tested whether symbiont-produced dsRNA can protect bees against *Varroa* mites. When *Varroa* mites parasitize bees, they feed on fat bodies (*18*) and ingest dsRNA present in that tissue, triggering their own RNAi response. Using mite RNAi to target essential mite genes results in mite death or lowered reproduction (*8*). We designed a dsRNA-producing plasmid with 14 concatenated sequences from essential genes previously shown to kill *Varroa* (pDS-VAR) (Fig. 4A and fig. S8) (*8*). We inoculated bees with *S. alvi* bearing pNR, pDS-GFP, or pDS-VAR; introduced adult *Varroa* mites 5 days later; and monitored mite survival for 10 days. Mites that fed on bees colonized with pDS-VAR

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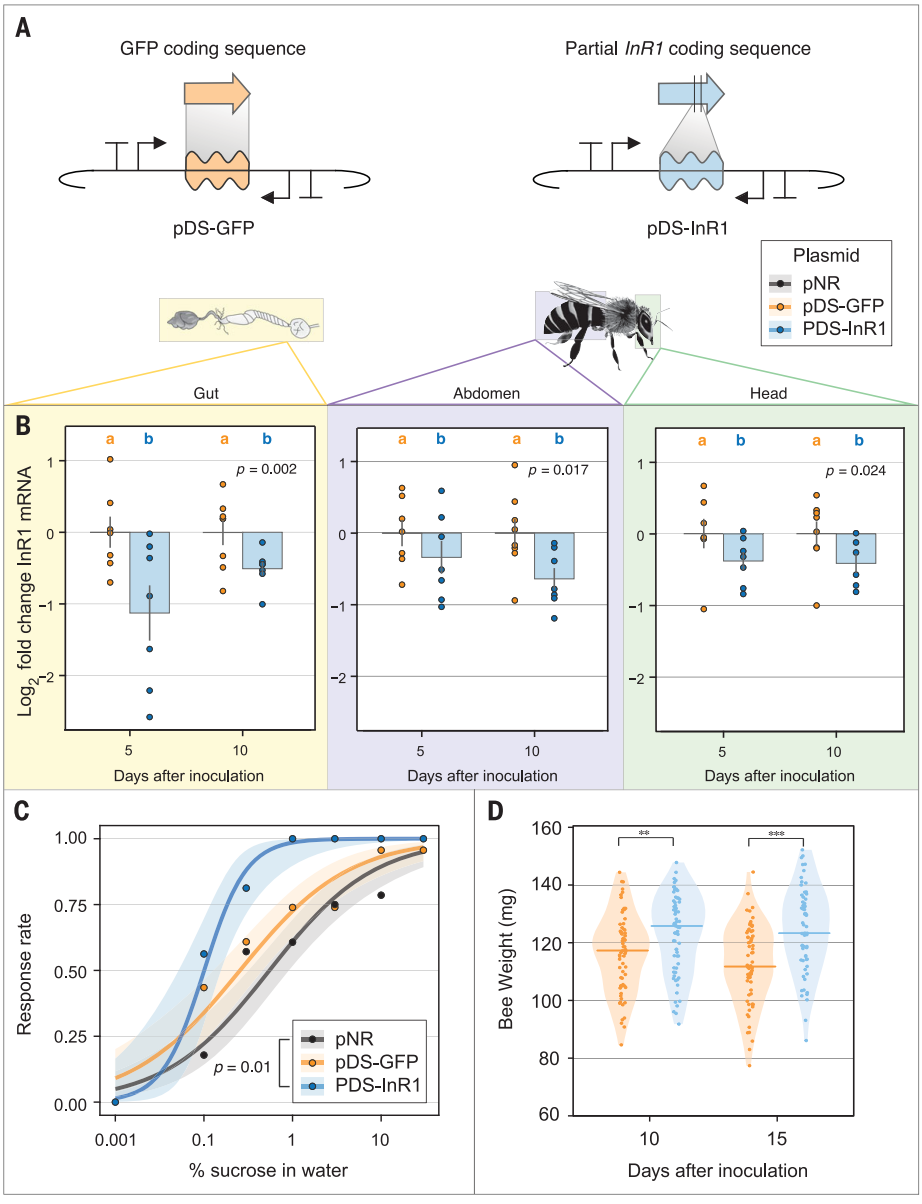

Fig. 2. Symbiont-mediated RNAi reduces expression of a specific host gene and alters feeding behavior and physiology. (**A**) Plasmid design for off-target dsRNA control plasmid (pDS-GFP) and *InR1* knockdown plasmid (pDS-InR1). (**B**) Bees colonized with engineered *S. alvi* expressing *InR1* dsRNA (pDS-InR1 plasmid) show reduced expression of *InR1* throughout body regions for 10 days compared to bees colonized with off-target dsRNA control (pDS-GFP). Total $N = 29$ bees from one hive. (**C**) pDS-InR1 plasmid increases host feeding activity (sucrose sensitivity response), measured 5 days after inoculation. Curves are a binomial family generalized linear model fit to the response data for $N = 67$ bees from two hives. (**D**) pDS-InR1 plasmid significantly increases bee weight, measured 10 and 15 days postinoculation (Mann-Whitney *U* test). Total $N = 135$ bees from one hive. See fig. S4 for data from an additional trial. Error bars and shading represent SEs. $^{**}P < 0.01$; $^{***}P < 0.001$.

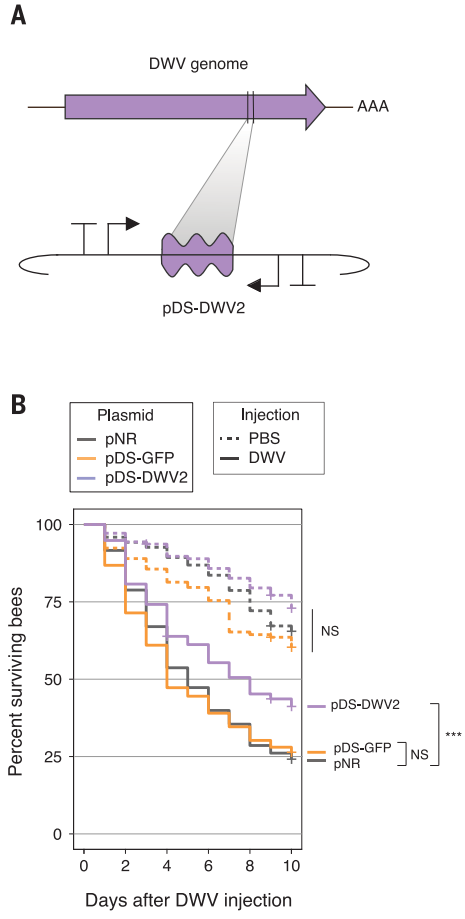

Fig. 3. Symbiont-produced RNAi can improve honey bee survival after viral injection. (**A**) Design of the DWV knockdown construct pDS-DWV2. (**B**) Survival curves of bees monitored for 10 days after injection with DWV or the phosphate-buffered saline (PBS) control. Bees inoculated with pNR, pDS-GFP, or pDS-DWV2 and then injected with PBS showed no significant change in survival (dotted lines). When injected with DWV, bees inoculated with pDS-DWV2 showed increased survival compared with bees inoculated with pNR (no dsRNA control) or pDS-GFP (off-target dsRNA control). $^{***}P < 0.001$ (Wald test); NS, not significant. Total $N = 980$ bees, sourced from three separate hives.

bacteria died more quickly than mites that fed on control bees (Fig. 4B).

Determining whether engineered symbiotic bacteria can improve whole hive health will require further testing. It is promising that inoculating bees with dsRNA-producing strains alone has no negative effect on their survival (fig. S9). Ongoing within-hive transmission could increase the effectiveness of this treatment by promoting the persistence and spread of engineered strains to new bees. Natural transmission of *S. alvi* and other bee gut symbionts occurs through direct social contact within hives (*15*), and engineered *S. alvi* strains are transferred between cohoused bees in the lab (fig. S10), suggesting that within-hive transmission is likely. Less is known about between-hive transmission of the bee gut microbiota. Use of this approach outside of the laboratory would require an understanding of these processes and the necessary biocontainment safeguards.

The degree of protection of bees that we observed in our experiments could likely be improved by further optimizing this symbiont-mediated RNAi delivery system. The specific dsRNA sequence chosen will affect the efficacy of targeted RNAi knockdown, as has been shown for suppression of DWV by oral delivery of RNAi (*19*). Engineering *S. alvi* to deliver more dsRNA to bees (e.g., by reducing

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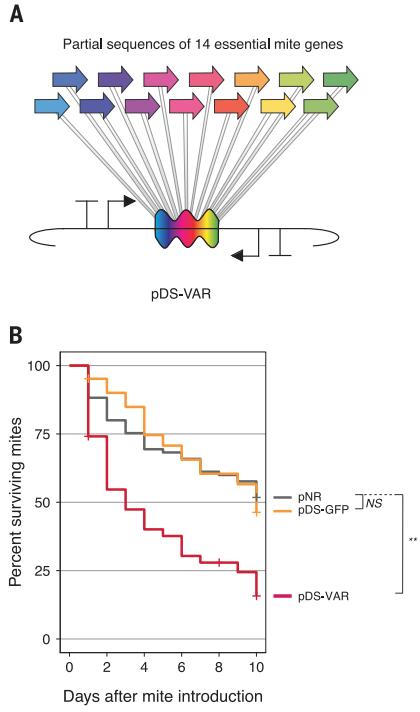

Fig. 4. Symbiont-produced RNAi kills *Varroa* mites feeding on honey bees. (**A**) Design of pDS-VAR plasmid targeting essential *Varroa* genes. (**B**) Survival curves for *Varroa* mites that fed on bees colonized with engineered *S. alvi*. Total $N = 253$ mites. All mites came from a single infested hive. Bees were sourced from three separate hives. $^{**}P < 0.01$ (Wald test); NS, not significant.

ribonuclease III activity) could also improve efficacy (*20*). The deleterious effects of *Varroa* mites and viruses for which the mites act as vectors are interdependent (*2*); both types of pests could be targeted simultaneously by symbiont-mediated RNAi, which might lead to synergistic improvements in bee health or more robust protection in the context of the fluctuating biotic interactions within hives. For example, co-infecting viruses that encode RNAi suppressors may limit the efficacy of symbiont-mediated RNAi (*21*); thus, a strategy that exploits the RNAi machinery of both bees and mites could ensure more consistent benefits to bee health.

We have shown that microbiome engineering can increase resistance to pathogens, a strategy proposed for humans (*22*) and honey bees (*23*, *24*). Insect-associated microbes have been engineered to interfere with mosquito transmission of malaria (*25*) and to kill crop pests (*26*), but not to improve pollinator health. Our results imply movement of symbiont-produced dsRNA from the gut lumen into bee cells but do not identify the mechanism of transfer. Possibly, lysis of *S. alvi* cells releases dsRNA to be taken up through the same route as orally administered dsRNA. Alternatively, symbiont-mediated dsRNA delivery may co-opt an uncharacterized interaction of *S. alvi* with its bee host, such as outer membrane vesicle production (*27*) or direct RNA export (*28*). Symbiont-mediated RNAi provides a new tool to study bee biology and to improve resilience against current and future challenges to honey bee health.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S11
Tables S1 to S4
References (*29*–*37*)

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Inducing immune bee genes

Honey bees are prone to parasitism by the *Varroa* mite, which is a vector for several bee pathogens. However, honey bees are also host to the symbiotic gut bacterium *Snodgrasella alvi.* Leonard *et al.* engineered *S. alvi* to produce double-stranded RNA (dsRNA)—a stimulus for insect RNA interference defense responses—from a plasmid containing two inverted promoters tagged with a fluorescent label (see the Perspective by Paxton). This dsRNA module can be targeted to interfere with specific bee genes as well as crucial viral and mite genes. The authors found that gene expression could be blocked for at least 15 days as the symbionts established in the bees' guts and continuously expressed the dsRNA constructs. *S. alvi* with specifically targeted plasmids not only suppressed infection with deformed wing virus but also effectively reduced *Varroa* mite survival.

Science, this issue p. 573; see also p. 504

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