A small RNA from a natural bacterial pathogen of *C. elegans* induces transgenerational inheritance of learned avoidance

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Summary

A small RNA from a clinical isolate of *Pseudomonas aeruginosa,* PA14, induces learned avoidance and its transgenerational inheritance in *C. elegans*. However, it is not known if small RNAs from bacteria found in *C. elegans'* natural habitat can regulate host behavior and produce heritable behavioral effects. Here we found that GRb0427, a pathogenic *Pseudomonas vranovensis* strain isolated from the *C. elegans* microbiota, similarly affects worm behavior: worms learn to avoid this pathogenic bacterium following exposure, and this learned avoidance is inherited for four generations. The learned response is entirely mediated by small RNAs, which induce avoidance and transgenerational inheritance in both the laboratory N2 strain and a natural isolate of *C. elegans*, JU1580, providing further support that such mechanisms of learning and inheritance exist in the wild. A small RNA, Pv1, matches the sequence of *C. elegans maco-1,* and is both necessary and sufficient to induce learned avoidance in worms. However, Pv1 also results in avoidance of a beneficial microbiome strain, *P. mendocina*; this maladaptive response may favor reversal of the transgenerational memory after a few generations. Our findings suggest that bacterial small RNA-mediated regulation of host behavior and its transgenerational inheritance are functional in *C. elegans'* natural environment, and that different bacterial small RNA-mediated regulation systems evolved independently but define shared molecular features of bacterial small RNAs that produce transgenerationally-inherited effects.

Introduction

Plants and animals have evolved diverse mechanisms to adapt to constantly changing environmental stimuli. Some of these stimuli are encoded as molecular changes that do not involve changes in DNA sequence, but are instead epigenetic, that is, mediated through changes in non-coding RNAs, DNA modifications, histone modifications, and nucleosome positioning (Brzezinka et al., 2016; Burton and Greer, 2022; Cecere, 2021; Liberman et al., 2019; Lim and Brunet, 2013; Sun et al., 2015). These changes can occasionally cross the germline and confer adaptive benefits to one generation of progeny (intergenerational) (Burton et al., 2021, 2020, 2017; Conine et al., 2018, 2013; Guida et al., 2019; Hibshman et al., 2016;

Hong et al., 2021; Jordan et al., 2019; Lim et al., 2021; Ow et al., 2021; Pereira et al., 2020; Perez et al., 2021; Willis et al., 2021) or more (transgenerational) (Gammon et al., 2017; Jobson et al., 2015; Kaletsky et al., 2020; Legüe et al., 2022; Mondotte et al., 2020; Moore et al., 2021, 2019; Rechavi et al., 2014, 2011; Toker et al., 2022; Vogt and Hobert, 2023; Wang et al., 2022; Webster et al., 2018). Multigenerationally-inherited effects can provide adaptive advantages in rapidly changing environments, particularly in organisms with short generation times (Baugh and Day, 2020; Sengupta et al., 2023; Uller et al., 2015).

Over the past decade, several instances of multigenerational inheritance have been reported in various organisms (Perez and Lehner, 2019; Quadrana and Colot, 2016; Santilli and Boskovic, 2023). Previous work from our lab characterized a striking example of epigenetic inheritance in response to a physiological stimulus, highlighting its adaptive benefits in *C. elegans*: upon exposure to the pathogenic *Pseudomonas aeruginosa* strain PA14, worms learn to subsequently avoid the bacteria, then pass on this learned avoidance to four generations of progeny (Moore et al., 2019). P11, a single small RNA from PA14, mediates this avoidance and its transgenerational inheritance through downregulation of the worm neuronal gene *maco-1* (Kaletsky et al., 2020; Moore et al., 2021). These studies provided the first example of bacterial small RNA-mediated regulation of a learned behavior (Kaletsky et al., 2020; Moore et al., 2021, 2019). However, PA14 is a human clinical *Pseudomonas* isolate; whether bacteria in *C. elegans'* natural environment elicit similar learned responses and multi-generational inheritance of learned responses through small RNAs is not known.

Diverse bacterial species influence *C. elegans* physiology and life history traits (Irazoqui et al., 2010; Pérez-Carrascal et al., 2022; Stuhr and Curran, 2020). Bacterial species from *C. elegans*' natural environment have been systematically characterized (Berg et al., 2016; Dirksen et al., 2016; Morgan et al., 2021; Petersen et al., 2022; Samuel et al., 2016; Yang et al., 2019; Zhang et al., 2021). These studies revealed multiple features of the microbiota in *C. elegans* natural habitat and their relationship to host physiology (Dirksen et al., 2020; Zhang et al., 2017). Studying bacterial species that are naturally associated with *C. elegans* might reveal processes that occur in the wild, and not in the laboratory, and vice versa; for example, bacteria from *C. elegans'* natural environment suppress mortal germline phenotypes that wild worms exhibit on laboratory strains of *E. coli* (Lise Frézal et al., 2023). Therefore, it is important to test the physiological relevance of laboratory experimental results under more natural conditions.

Bacterial species in the worm microbiome that induce stress and immune response reporters are categorized as pathogenic (Samuel et al., 2016), while species that serve as sources of nutrition and increase growth rates are categorized as beneficial (Haçariz et al., 2021; Samuel et al., 2016; Zhang et al., 2021), and some of these species confer protection against pathogenic species (Dirksen et al., 2016; Kissoyan et al., 2019). Other species are beneficial in some contexts and pathogenic in others (Kissoyan et al., 2022). Therefore, it may be evolutionarily favorable for worms to have plastic responses to different bacterial classes that they naturally encounter. Worms are naively attracted to specific beneficial and neutral bacterial species (e.g., *Pseudomonas mendocina* and *Proteus mirabilis*, respectively) when given a choice between these bacteria and their laboratory diet *E. coli* HB101 (Chen et al., 2022). Similarly, worms grown on the beneficial bacterial strain *Providentia alcalifaciens* prefer this bacterial species over their laboratory diet *E. coli* OP50 in a

behavioral choice assay (O'Donnell et al., 2020). These beneficial bacterial species modulate *C. elegans'* attraction towards several chemicals. Naïve or learned attraction in response to beneficial bacteria that worms encounter in their natural environment may have evolved as an evolutionarily favorable strategy. However, it is not known if worms can learn to avoid the pathogenic bacterial species in their environment. Additionally, whether bacteria in *C. elegans'* natural environment can modulate the host nervous system through small RNAs and whether they can induce transgenerationally-inherited effects are not known.

Pseudomonas is the largest among the bacterial genera that constitute *C. elegans'* natural microbiome (Samuel et al., 2016). In this study, we examined *C. elegans'* behavioral responses to a Pseudomonad species present in its natural microbiome. We found that an isolate of *Pseudomonas vranovensis* can elicit learned avoidance and its transgenerational inheritance through a single small RNA that is both necessary and sufficient. However, this learned response to *P. vranovensis* also leads to avoidance of a beneficial bacteria also found in *C. elegans'* environment, *P. mendocina*. Our work reveals a transgenerational effect in response to bacteria in *C. elegans'* natural microbiome, underscoring the physiological relevance of transgenerational inheritance and its significance in the wild. We also identified a new small RNA that can induce a learned behavior in *C. elegans*, therefore expanding the repertoire of bacterial small RNA-mediated regulation of the host nervous system and helping to identify characteristics of small RNAs necessary for trans-kingdom signaling. Finally, the induced avoidance of a beneficial bacteria after pathogen training suggests that "forgetting" learned pathogen avoidance after a few generations might benefit *C. elegans*, limiting maladaptive behaviors.

Results

C. elegans is attracted to the natural bacterial pathogen, *P. vranovensis*

To examine if exposure to bacteria from *C. elegans'* natural environment can produce stereotypic behavioral responses and whether these could potentially be small RNA-mediated, we were curious about *C. elegans'* response to strains that are present in its natural microbiome (Samuel et al., 2016); one such strain, GRb0427, is an isolate of *Pseudomonas vranovensis.* Prior to exposure to this pathogen, worms strongly prefer GRb0427 relative to their laboratory food *E. coli* OP50 (naïve, **Figure 1A**). This preference likely evolved as *Pseudomonas* bacteria are sources of nutrition to *C. elegans* in the wild (Samuel et al., 2016). Despite worms' attraction to GRb0427, this bacterium is pathogenic to *C. elegans*: adult exposure to GRb0427 causes severe illness (**Figure 1B**) and significantly reduces survival to less than 2-3 days (**Figure 1C**), in contrast to *C. elegans'* normal lifespan of 2-3 weeks.

C. elegans learn to avoid *P. vranovensis* after exposure

Upon cultivation on a GRb0427 lawn for 24 hours, worms learn to robustly avoid the bacteria, as shown in a choice assay between OP50 and GRb0427 (**Figure 1D**). To determine if the learned *P. vranovensis* avoidance reflects a choice made by *C. elegans* in the wild, we tested a natural isolate of *C. elegans*, for its response to *P. vranovensis.* JU1580 has a similar naïve preference to *P. vranovensis* as the laboratory strain, N2 (**Fig. 1E**). Like N2, JU1580 learns to avoid *P. vranovensis* GRb0427 upon a 24-hour exposure to a GRb0427 bacterial lawn (**Figure 1F**).

Exposure to *P. aeruginosa* PA14 causes gene expression changes in specific *C. elegans* sensory neurons; specifically, a 24-hour exposure to PA14 results in the induction of *daf-7p::gfp* expression in the ASJ

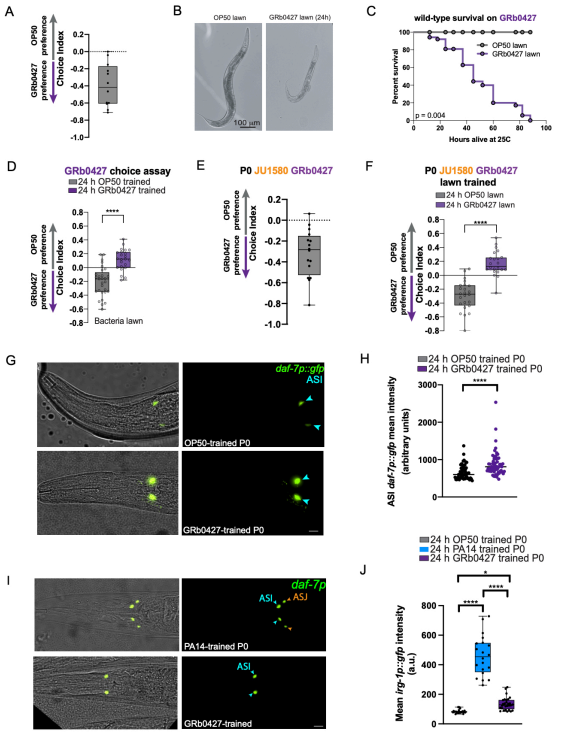

Figure 1. GRb0427, a natural Pseudomonad pathogen of *C. elegans* induces learned avoidance. (A) Untrained worms prefer *P. vranovensis* GRb0427 over their bacterial food *E. coli* OP50 in a bacterial choice assay (see Methods, Choice index = (number of worms on OP50 – number of worms on GRb0427)/ (total number of worms)). (B) Representative images acquired after exposing Day 1 worms to OP50 (left) and GRb0427 (right) for 24 hours. GRb0427 is pathogenic and 24-hour exposure makes worms sick. (C) Worms have significantly lower survival on a GRb0427 lawn (compared to an OP50 lawn). (D) Worms learn to avoid GRb0427 following 24-hour exposure. (E) Untrained JU1580 (a natural isolate of *C. elegans*) worms are also attracted to GRb0427 relative to OP50. (F) JU1580 worms also learn to avoid GRb0427 after a 24-hour exposure to a GRb0427 lawn. (G) Representative image of a 24-hour OP50-trained (top) or GRb0427-trained worm (bottom) expressing *daf-7::GFP. daf-7::GFP* is expressed in the ASI sensory neurons. (right panel, blue arrowheads). Left panel is a merge of the GFP channel (right) and a brightfield channel. Scale bar = 10 µm. (H) Quantification of mean ASI *daf-7p::gfp* intensities from OP50 and GRb0427 animals shows higher expression in GRb0427-trained animals. (I) Representative image of a 24-hour PA14-trained (top) or GRb0427-trained (bottom) worm. *daf-7::GFP* is expressed in the ASI (blue arrowheads) and ASJ (orange arrowheads) sensory neurons (right panel). Left panel is a merge of the GFP channel (right) and a brightfield channel. Scale bar = 10 µm. (J) Mean fluorescence intensity of an *irg-1p::gfp* innate immune response reporter in OP50 (gray), *Pseudomonas aeruginosa* PA14 (blue), and GRb0427 (purple)-trained worms. Mean *irg-1::gfp* reporter intensity is significantly lower in GRb0427-trained worms compared to PA14-trained worms. Each dot represents an individual choice assay plate (A, D-F) or an individual neuron (for fluorescence images) (H,J). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test, ****p < 0.0001 (D, H); one-way ANOVA with Tukey's multiple comparison's test, ****p<0.0001, *p < 0.05 (J).

neurons and an increase in *daf-7p::gfp* expression in the ASI neurons (Meisel et al., 2014; Moore et al., 2019). PA14 small RNAs induce expression of *daf-7p::gfp* in the ASI, which persists in the F1-F4 progeny generations (Kaletsky et al., 2020; Moore et al., 2019), while the increase in ASJ *daf-7* is caused by PA14 secondary metabolites phenazine-1-carboxamide and pyochelin (Meisel et al., 2014), and does not persist beyond the P0 (Moore et al., 2019). To determine whether altered *daf-7* levels underlie the learned avoidance response to *P. vranovensis*, we examined *daf-7p::gfp* expression in GRb0427-trained animals (P0). Upon GRb0427 exposure, *daf-7p::gfp* levels significantly increase in the ASI neurons (**Figure 1G-H)**, but no expression was observed in the ASJ neurons (**Figure 1G**), in contrast to the response to PA14 training (**Figure 1I**). Unlike PA14 and other pathogenic bacteria, exposure to GRb0427 triggers a significantly milder innate immune response, as indicated by low expression of a reporter of the *irg-1* gene reporter (**Figures 1J, S1**). Lack of induction of phenazine-mediated ASJ *daf-7p::gfp* expression and only a mild induction of the *irg-1* dependent innate immune pathway suggested that these innate immune pathways might not play a significant role in the neural response to GRb0427, even in the P0 generation.

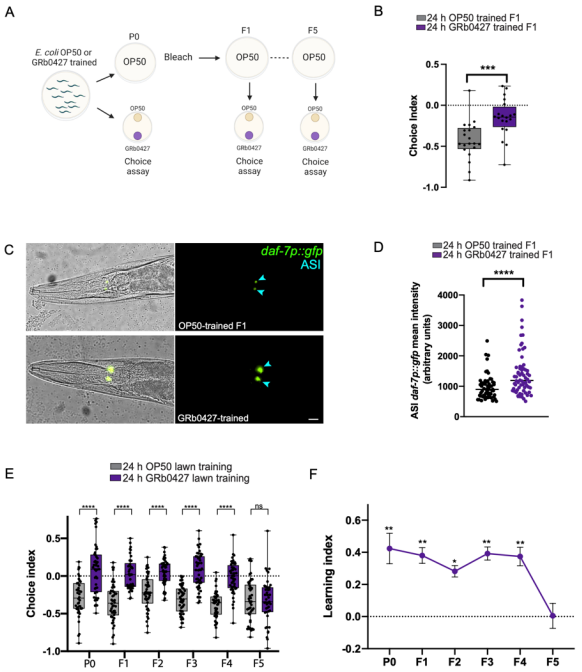

Figure 2. GRb0427-mediated learned avoidance is inherited transgenerationally

(A) Worms trained for 24 hours on *E. coli* OP50 or GRb0427 are bleached to obtain eggs, which are allowed to grow to Day 1 adults on OP50 plates. These adult F1 progeny are tested in a choice assay between OP50 and GRb0427. Some of the adult F1s are bleached to obtain F2 progeny that are again grown to Day 1 and tested in a choice assay. This is continued till the fifth progeny generation (F5). (B) F1 progeny of GRb0427-trained worms avoid GRb0427 compared to F1 progeny of OP50-trained worms. (C) Representative images of F1 progeny obtained from an OP50-trained worm population (top) and a GRb0427-trained worm population (bottom). F1 progeny of GRb0427-trained animals have higher *daf-7p::gfp* expression in the ASI sensory neurons (right panel, blue arrowheads). Left panels are merged images of the GFP channel (right) and a brightfield channel. Scale bar = 10 µm. (D) Quantification of mean ASI *daf-7p::gfp* intensities from F1 progeny of OP50-trained and GRb0427 animals shows higher expression in F1 progeny of GRb0427-trained animals. (E) Untrained F1-F4 progeny of GRb0427-trained P0 animals avoid GRb0427 relative to the progeny of OP50-trained control P0 animals. This avoidance is lost in the F5 generation. (F) Learning index (naive choice index – trained choice index) of generations P0–F5. Error bars represent mean ± SEM. Each dot represents an individual choice assay plate (B,E) or an individual neuron for fluorescence images (D). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test, ****p < 0.0001, ***p<0.001; one-way ANOVA with Tukey's multiple comparison's test, ****p<0.0001, **p<0.01, *p<0.05, ns, not significant (E, F).

The avoidance response to *P. vranovensis* is transmitted for four generations

The learned avoidance induced by a PA14 lawn and the PA14 small RNA P11 are communicated to naïve progeny (Moore et al., 2019), and *daf-7* levels are also increased in the ASI neurons in the parental and four subsequent generations (Moore et al., 2019). Since training on *P. vranovensis* resulted in an increase in *daf-7p::gfp* levels in the ASI, we hypothesized that the learned avoidance to GRb0427 might be inherited by the progeny of trained mothers. Indeed, the adult F1 progeny of GRb0427-trained mothers showed robust avoidance of *P. vranovensis* compared to the F1 progeny of the control (OP50-trained) mothers (**Figure 2A, B**). Additionally, F1 progeny of GRb0427-trained mothers have higher *daf-7p::gfp* levels in the ASI neurons compared to that in F1 animals from OP50-trained mothers (**Figure 2C-D**), similar to their levels in P0 animals (**Figure 1G-I**).

To examine if learned avoidance to GRb0427 is inherited beyond the F1 generation, we tested the F2-F5 progeny (**Figure 2A**); the learned avoidance of GRb0427 lasts up to the F4 generation, but returns to naïve attraction to GRb0427 in the F5 generation (**Figure 2E, F**). Thus, GRb0427 training does induce trans-generational inheritance of learned *P. vranovensis* is constant across P0 through F4 (Figure 2E-F), consistent with the *daf-7p::gfp* and *irg-1p::gfp* results (Figure 1G-J), suggesting that innate immunity pathways do not contribute significantly to *C. elegans'* avoidance of *P. vranovensis*.

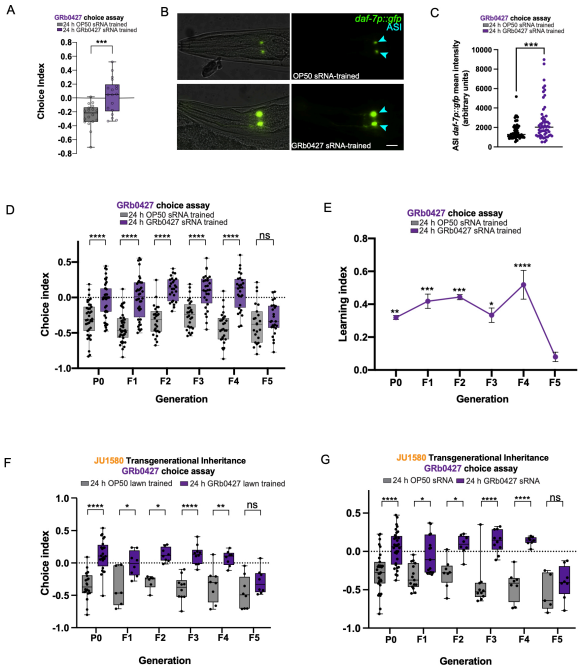

Figure 3. GRb0427 small RNAs induce learned avoidance and its transgenerational inheritance (A) Worms trained on GRb0427 small RNAs exhibit learned avoidance of GRb0427 in an OP50-GRb0427 choice assay. (B) Representative images of an OP50 sRNA-trained worm (top) and a GRb0427 sRNA-trained worm (bottom). GRb0427 sRNA-trained animals have higher *daf-7p::gfp* expression in the ASI sensory neurons (right panel, blue arrowheads). Left panels are merged images of the GFP channel (right) and a brightfield channel. Scale bar = 10 µm. (C) Quantification of mean ASI *daf-7p::gfp* intensities from OP50 sRNA-trained and GRb0427 sRNA-trained animals shows higher expression in GRb0427 sRNA-trained animals. (D) Untrained F1-F4 progeny of GRb0427 sRNA-trained P0 animals avoid GRb0427 relative to the progeny of OP50 sRNA-trained control P0 animals. This avoidance is lost in the F5 generation. (E) Learning index (naive choice index – trained choice index) of generations P0–F5. Error bars represent mean ± SEM. (F) Untrained F1-F4 progeny of GRb0427-trained P0 JU1580 (a natural *C. elegans* isolate) animals avoid GRb0427 relative to the progeny of OP50-trained control P0 JU1580 animals. This avoidance is lost in the F5 generation. (G) GRb0427 sRNA-trained JU1580 animals and their F1-F4 progeny avoid GRb0427 relative to the respective controls. This avoidance is lost in the F5 generation. Each dot represents an individual choice assay plate (A, D, F, G) or an individual neuron for fluorescence images (C). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test (A, C), ***p<0.001; one-way ANOVA with Tukey's multiple comparison's test, ****p < 0.0001, ***p<0.001, **p<0.01, *p<0.05, ns, not significant (D, E, F, G).

P. vranovensis small RNAs drive learned avoidance

Since learned avoidance to *P. vranovensis* is transgenerationally inherited, as we previously observed with PA14 training, we next asked if small RNAs made by *P. vranovensis* induce avoidance, as is the case for PA14 (Kaletsky et al., 2020). When adult *C. elegans* were exposed for 24 hours to sRNAs isolated from *P. vranovensis*, worms learned to avoid *P. vranovensis* (**Figure 3A**). Exposure to *P. vranovensis* sRNAs increased *daf-7p::gfp* expression in the ASI sensory neurons (**Figure 3B, C**). We next tested if *P. vranovensis* sRNA-induced learned avoidance is transgenerationally inherited. Indeed, as observed for *P. vranovensis* lawn exposure, *P. vranovensis* sRNA-induced avoidance is inherited up to the F4 generation and resets in the F5 (**Figure 3D, E**).

Like the lab-bred strain N2, the wild *C. elegans* isolate JU1580 can also learn avoidance of *P. vranovensis* upon exposure to *P. vranovensis* small RNAs, and this learned avoidance persists for four generations (**Figure 3F, G**).

A specific *P. vranovensis* sRNA induces learned avoidance

We next asked if learned avoidance induced by *P. vranovensis* small RNAs is species-specific. We trained worms on *P. vranovensis* sRNAs and tested avoidance of PA14; *P. vranovensis* sRNA training induces avoidance to PA14, similar to training on PA14 sRNAs (**Figure 4A**). Intriguingly, worms exposed to bacteria expressing only the PA14 sRNA P11 also induces avoidance to *P. vranovensis* (**Figure 4B**), suggesting that the underlying mechanism in *P. vranovensis* sRNA-induced avoidance is like

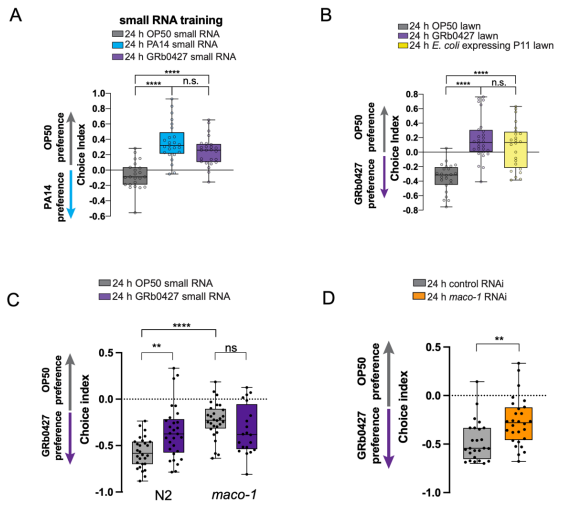

Figure 4. GRb0427 sRNA-induced avoidance requires *maco-1* (A) Worms were trained on OP50 (gray), *Pseudomonas aeruginosa* PA14 (blue), or GRb0427 (purple) small RNAs, and tested for their PA14 preference in a bacterial choice assay between OP50 and PA14. Worms treated on GRb0427 small RNAs not only avoid GRb0427 (Figure 3A), but also avoid PA14 in an OP50-PA14 choice assay. (B) Worms trained on the PA14 small RNA P11 avoid GRb0427 in an OP50-GRb0427 choice assay. (C) N2 (wild type) worms trained on GRb0427 small RNAs avoid GRb0427. *maco-1(ok3165)* loss-of-function mutant worms naively avoid GRb0427 and do not show increased avoidance upon exposure to GRb0427 sRNAs. (D) Upon downregulation of *maco-1* by RNAi, wild type worms exhibit higher avoidance of GRb0427 compared to control RNAi-treated wild type worms. Each dot represents an individual choice assay plate (A-D). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values, One-way ANOVA with Tukey's multiple comparison's test, ****p<0.0001, ns, not significant (A,B); Two-way ANOVA with Tukey's multiple comparison's test, **p<0.01, ****p<0.0001, ns, not significant (C); Unpaired, two-tailed Student's t test, **p<0.01 (D).

that of PA14-induced avoidance. Consistent with this idea, worms with a loss-of-function mutation in *maco-1*, the *C. elegans* gene targeted by P11, naively avoid *P. vranovensis,* and exposure to *P. vranovensis* small RNAs does not further increase *P. vranovensis* avoidance (**Figure 4C**). Treatment with *maco-1* RNAi also increases naïve avoidance of GRb0427 (**Figure 4D**). This observation indicates that learned avoidance induced by *P. vranovensis* may target *maco-1,* as PA14's P11 small RNA does.

We next examined whether *P. vranovensis* might encode a P11-like small RNA. We analyzed the recently-sequenced genome of *P. vranovensis* (Burton et al., 2020) and did not find any region with sequence homology to P11. In fact, there is no region analogous to the operon that contains P11 in the *P. vranovensis* genome. That is, while P11 can induce similar avoidance of GRb0427 as it does for PA14, and GRb0427 induces avoidance through a small RNA, GRb0427 does not appear to have a small RNA with a P11-like sequence in its genome; therefore, we needed to identify a different sRNA in GRb0427 that induces learned avoidance and inheritance of this avoidance.

While a P11-like sRNA is not required for the learned avoidance of GRb0427, our small RNA *maco-1* experiments suggested that an sRNA with similarity to *maco-1* might be involved. Therefore, we searched the *P. vranovensis* genome for *maco-1* sequence similarity; we identified an intergenic region with 16 nucleotides of perfect sequence identity to the *maco-1* gene (**Figure 5A**). Interestingly, this sequence identity lies in a different *maco-1* exon from P11's 17nt perfect match. The *P. vranovensis* intergenic region containing this 16-nucleotide sequence match to *maco-1* is flanked by bacterial protein coding genes with predicted functions in the iron metabolism and sugar transport pathways (**Figure 5B**). We next asked if exposing worms to *E. coli* bacteria expressing only this intergenic region would induce avoidance. Indeed, *E. coli* expressing 347 bp of this intergenic region induces avoidance to *P. vranovensis* (**Figure 5C**). The avoidance induced by *E. coli* expressing the intergenic region persists for four

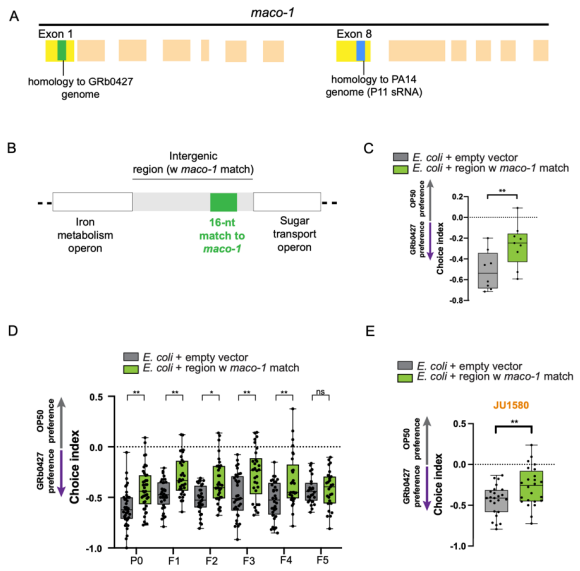

Figure 5. An intergenic region in the GRb0427 genome contains a 16-nucleotide perfect match to *maco-1* and is sufficient for learned avoidance of GRb0427. (A) The PA14 small RNA P11 contains a 17-nucleotide perfect match to the worm neuronal gene *maco-1* (exon 8). By annotating the GRb0427 genome, we discovered an intergenic region with a 16-nucleotide perfect match to a stretch of exon 1 of *maco-1.* (B) The GRb0427 genome has an intergenic region (flanked by an iron metabolism operon and a sugar transport operons) containing a 16-nucleotide perfect match to *maco-1.* (C) Training worms on *E. coli* expressing the intergenic region (with the match to *maco-1*) induces avoidance of GRb0427. (D) Untrained F1-F4 progeny of worms trained on *E. coli* expressing the intergenic region (with the match to *maco-1*) exhibit higher avoidance of GRb0427 compared to controls. This higher avoidance is lost in the F5 generation. (E) Training of JU1580 worms on *E. coli* expressing the intergenic region (with the match to *maco-1*) induces avoidance of GRb0427. Each dot represents an individual choice assay plate (C-E). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test, $^{**}p < 0.01$, (C, E); one-way ANOVA with Tukey's multiple comparisons test, $^{**}p < 0.01$, $^{*}p<0.05$ (D), ns, not significant.

generations after parental exposure (**Figure 5D**), and is lost by the F5 generation, similar to transgenerational inheritance of learned avoidance induced by a *P. vranovensis* lawn or small RNA exposure. *E. coli* expressing the intergenic region with the match to *maco-1* also induces avoidance of GRb0427 in the wild worm isolate JU1580 (**Figure 5E**).

We next investigated if the intergenic region encodes a small RNA. We sequenced the *P. vranovensis* total small RNA pool that induces learned avoidance and its transgenerational inheritance. Indeed, we detected a small RNA within the intergenic region that contains the 16-nucleotide sequence match to *maco-1* (**Figure 6A, B)**. This small RNA, which we named Pv1**,** is predicted to be 121 bp long. Exposure to *E. coli* expressing Pv1 is sufficient to induce avoidance to *P. vranovensis* in the mother generation (**Figure 6C**), as well as the transgenerational inheritance of this learned avoidance (**Figure 6D**).

To determine if Pv1's sequence identity to *maco-1* is necessary for the learned avoidance to *P. vranovensis* and its transgenerational inheritance, we constructed a mutant *P. vranovensis* bacterial strain lacking the 16 nt match to *maco-1,* Δ16 (**Figure 6E**). Although this Δ16 mutant GRb0427 strain "smells" the same to worms as wild-type GRb0427 (**Figure 6F**) and is similarly pathogenic to worms as wild-type GRb0427 **(Figure 6G)**, Δ16 is unable to induce transgenerational inheritance of learned avoidance (**Figure 6H)**. Together, our data suggest that a specific *P. vranovensis* small RNA, Pv1, is necessary and sufficient for sRNA-mediated learned avoidance of GRb0427 and its transgenerational inheritance.

C. elegans exhibit maladaptive avoidance of the beneficial bacterium *Pseudomonas mendocina* following exposure to GRb0427 or Pv1

Transgenerational inheritance of learned avoidance of both PA14 and GRb0427 lasts for four generations, suggesting that there may be some benefit to "forgetting" learned

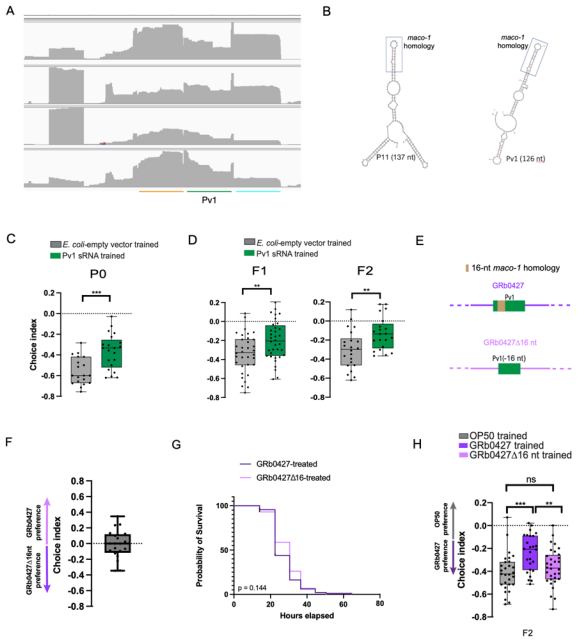

Figure 6. Pv1, a GRb0427 sRNA, is necessary and sufficient for transgenerational inheritance of learned avoidance (A) Alignment of GRb0427 small RNA sequencing reads to the GRb0427 genome. The peaks obtained from this alignment and the Sapphire small RNA promoter prediction software indicated the presence of 3 small RNAs (shown with blue, green and orange lines) in the intergenic region described in Figure 5. Of the 3 predicted sRNAs, the sRNA marked in green contains the 16-nucleotide match to *maco-1,* and we named it Pv1. (B) mFold structure predictions for P11 and Pv1 show striking similarities in secondary structure, and presence of a stem loop in the *maco-1* match region (the boxed regions) for both small RNAs. (C) Training worms on *E. coli* expressing just the Pv1 small RNA induces avoidance of GRb0427 (compared to training on *E. coli* expressing a control empty vector). (D) *E. coli*-Pv1 induced learned avoidance of GRb0427 is transgenerationally inherited. Untrained F1 and F2 progeny of *E. coli*-Pv1 trained P0 worms also exhibit higher GRb0427 avoidance compared to controls. (E) GRbΔ16nt is a mutant GRb0427 bacterial strain lacking the 16-nucleotide match to *maco-1* (constructed using two-step allelic exchange). (F) GRbΔ16nt 'smells' similar to wild type GRb0427, as naïve worms do not exhibit a preference towards either strain in a choice assay. (G) Survival of worms on lawns of GRbΔ16nt and wild type GRb0427 are not significantly different. (H) F2 progeny of GRbΔ16nt-trained worms do not exhibit learned avoidance of GRb0427. Each dot represents an individual choice assay plate (C, D, F, H). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. Unpaired, two-tailed Student's t test, $^{**}p < 0.01$, $^{***}p<0.001$, ns, not significant (C, D); One-way ANOVA with Tukey's multiple comparison's test, $^{**}p < 0.01$, $^{***}p<0.001$, ns, not significant (H). For the survival assay in (G), ns – not significant (by Log-rank (Mantel-Cox) test for survival).

avoidance of a pathogen. In the wild, worms experience a range of temperatures, and bacterial species can be differentially pathogenic at different temperatures. In the case of *P. aeruginosa*, pathogenicity decreases at lower temperatures (Kaletsky et al., 2020; Tan et al., 1999); thus, continuing to avoid this bacteria even at lower temperatures might cause the worms to mistakenly avoid a potentially good food source, and the PA14 small RNA, P11 is not expressed at lower temperatures (Kaletsky et al., 2020). We wondered if this was also the case for GRb0427 and Pv1, or if other factors, including other members of the microbiome, might influence the cessation of avoidance.

We first asked if temperature affects the pathogenicity of GRB0427. Although less pathogenic to worms than 25°C-grown GRb0427, 15°C-grown GRb0427 still kills all the worms in the population before the control (OP50)-grown worms have started dying (**Figure 7A**). The Pv1 small RNA is expressed in total RNA pools from both 25°C GRb0427 and 15°C GRb0427 (**Figure S2**). Consistent with Pv1 being expressed under both temperature conditions, worm populations trained on 25°C and 15°C GRb0427 small RNAs both avoid GRb0427 compared to worms trained on control (OP50) small RNAs (**Figure 7B**).

We next asked if GRb0427-induced learned avoidance might alter worms' responses to other bacteria. Several bacterial species in the worm microbiome are beneficial; that is, they increase worm growth rates and progeny production and extend

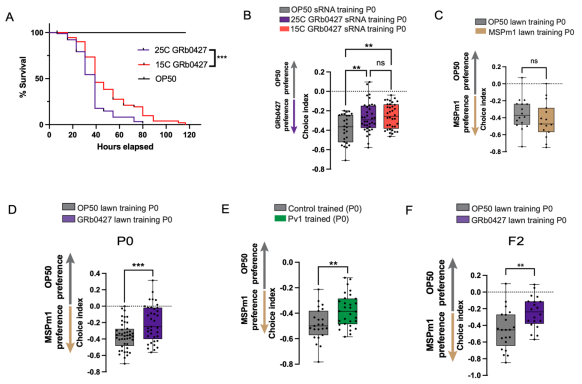

Figure 7. Pv1-induced learned avoidance causes avoidance of *P. mendocina*, a beneficial natural bacterial species. (A) Worms have significantly lower survival on a 25°C-grown GRb0427 lawn compared to a 15°C-grown GRb0427 lawn but in both these conditions, all worms die before worms on an OP50 lawn have started dying, indicating that both 25°C and 15°C-grown GRb0427 are pathogenic. (B) Training worms on sRNAs from both 25°C and 15°C-grown GRb0427 induce learned avoidance. (C) Training worms on a lawn of the beneficial bacterial species, *P. mendocina*, does not induce avoidance. (D) Training worms on a GRb0427 lawn reduces attraction to *P. mendocina* compared to OP50-trained control worms. (E) Training worms on *E. coli* expressing Pv1 reduces attraction to *P. mendocina.* (F) Naïve F2 progeny of GRb0427-trained P0 animals exhibit reduced attraction to *P. mendocina* relative to F2 progeny of OP50-trained P0 animals.Each dot represents an individual choice assay plate (C-F). Boxplots: center line, median; box range, 25th–75th percentiles; whiskers denote minimum-maximum values. One-way ANOVA with Tukey's multiple comparison's test, $^{**}p < 0.01$, ns, not significant; Unpaired, two-tailed Student's t test, $^{**}p < 0.01$, $^{***}p<0.001$, ns, not significant. For the survival assay in (A), $^{***}p<0.001$ (by Log-rank (Mantel-Cox) test for survival).

lifespan (Samuel et al., 2016). Therefore, weasked if exposure to the pathogenic GRb0427 might cause worms to inadvertently avoid beneficial bacteria in their microbiome. Some of the beneficial bacterial species in the worm microbiome, e.g., *Pseudomonas mendocina,* belong to the same genus as GRb0427 (Dirksen et al., 2020)*; C. elegans* are attracted to *Pseudomonas mendocina* (Chen et al., 2022). We found that worms trained on a strain of *P. mendocina* from the worm's microbiome, MSPm1, do not exhibit an altered response to *P. mendocina* compared to control (*E. coli* OP50)-trained worms (**Figure 7C**), as might be expected for beneficial bacteria. Consistent with this logic, we found that the *P. mendocina* genome does not encode for the Pv1 small RNA used by GRb0427 to elicit avoidance, and the intergenic regions in the genome do not contain any significant contiguous matches to the Pv1 target *maco-1*.

We next tested the effect of GRb0427 training on worms' responses to *P. mendocina*; worms exposed to GRb0427 have significantly reduced attraction to *P. mendocina* compared to worms grown on the control *E. coli* OP50 (**Figure 7D**). Similarly, training on Pv1 small RNA is sufficient to reduce attraction of worms to the beneficial *P. mendocina* (**Figure 7E**). Since Pv1 mediates transgenerational inheritance of learned GRb0427 avoidance, we next tested if altered *P. mendocina* preference upon GRb0427 training is transgenerationally inherited. Indeed, F2 progeny of GRb0427-trained animals exhibit reduced attraction to *P. mendocina* **(Figure 7F)**.

Taken together, our results suggest that in the wild, Pv1 small RNA-mediated learned avoidance may protect worms and their progeny from the pathogenic GRb0427, but since this also results in avoidance of beneficial bacteria, it may be beneficial to reverse this memory after a few generations when the worms may no longer encounter the original pathogen.

Discussion

C. elegans' wild environments are rife with diverse bacterial species, and recent studies have provided comprehensive descriptions of *C. elegans'* natural microbial environments (Berg et al., 2016; Dirksen et al., 2016; Samuel et al., 2016). The effects of

these bacterial species on *C. elegans'* nervous system and behavior have been largely unexplored, and whether these bacteria can exert transgenerational effects on worms was previously unknown. Here we show that a small RNA from GRb0427, a pathogenic bacterial species found in *C. elegans'* natural environment, induces learned avoidance in worms. This learned avoidance persists for four generations, similar to the learned avoidance induced by the P11 small RNA expressed in the clinical isolate of *P. aeruginosa*, PA14. The Pv1 small RNA, like P11, regulates avoidance by targeting the neuronal gene *maco-1* and subsequently upregulating the expression of the TGF-beta ligand *daf-7*. Our data suggest that the previously-described molecular mechanisms underlying PA14 sRNA-induced avoidance may also be employed to induce learned avoidance of pathogens in *C. elegans'* natural settings.

This wild bacterial species, *P. vranovensis*, however, does not express the P11 small RNA; instead, it uses a distinct small RNA, which we identified and named Pv1, to induce avoidance and its transgenerational inheritance. These findings establish bacterial small RNA-mediated regulation of host physiology as a phenomenon that may occur in the wild and in *C. elegans'* interaction with multiple different bacterial species. In fact, our data indicate that small RNAs may play a significant role in the neuronal response of *C. elegans* to wild bacteria: the behavioral response to GRb0427 is largely small RNA-dependent. In fact, the secondary metabolite-induced neuronal response observed upon *Pseudomonas aeruginosa* PA14 exposure (*daf-7* induction in ASJ neurons) (Meisel et al., 2014; Moore et al., 2019) is not observed upon GRb0427 exposure, suggesting that the sRNA-mediated response may be the major mechanism of avoidance of this pathogen. The sRNA-mediated learned response is also mechanistically distinct from a previously-described intergenerational effect on progeny survival after *P. vranovensis* exposure (Burton et al., 2020).

Our study provides an example of small RNA-mediated cross-kingdom signaling between bacteria and their animal hosts, adding to the diverse instances of RNA-based trans-kindom signaling (Kaletsky et al., 2020, Weiner et al. 2013, Chow et al., 2019). The identification of Pv1 expands the repertoire of bacterial small RNAs that can affect host physiology and begins to identify common principles of bacterial small RNA-mediated regulation of *C. elegans* behavior. Both Pv1 and P11 target *maco-1*, a conserved ER-localized calcium regulator (Arellano-Carbajal et al., 2011), indicating that the sRNA/*maco-1/daf-7* axis may be important for regulation by multiple bacterial small RNAs. Pv1, like P11, has a perfect match to its putative target, *maco-1,* indicating that bacterial small RNAs require perfect sequence complementarity for sRNA-mediated effects; moreover, these matches are present in stem loops of predicted secondary structures of these sRNAs (**Figure 6B**). However, Pv1 and P11 contain sequence matches to different exons of the *maco-1* gene sequence (**Figure 5A**), suggesting that the P11-matched region of *maco-1* is not the key element. Although P11 and Pv1 are similar in secondary structure and length, they have very low sequence homology, suggesting that these two small RNAs likely evolved independently. The fact that the *maco-1* sequence matches are present in stem loops may also suggest that there may be convergent evolution towards a particular length or secondary structure. This supports the idea that several other bacterial sRNA-based regulation systems exist, that may target the same or distinct *C. elegans* genes, and may be yet to be discovered. Characterizing sRNAs from additional bacterial species that induce heritable behaviors in worms may help define molecular and

structural features of sRNAs that can induce learning and inheritance, and may even allow us to predict which naturally existing exogenous sRNAs, including sRNAs from animal gut microbiomes, have the potential to induce heritable effects.

We showed that exposure to *P. vranovensis* GRb0427 alters *C. elegans'* response to multiple bacterial species; not only do they learn to avoid the pathogenic GRb0427, but they also (mistakenly) avoid the beneficial *P. mendocina.* In the wild, it might be beneficial to inherit this transgenerational memory for only a limited number of generations, rather than indefinitely, to prevent the deleterious avoidance of nutrient sources. After exposure to a pathogen such as GRb0427, learned avoidance memory might protect the next few progeny generations from the pathogen, but it might be beneficial to revert this memory, since the environmental microbial composition in the wild may subsequently change to include beneficial bacteria. Reversal of this memory may have evolved to protect worms from maladapttive avoidance of other beneficial bacterial species once the pathogen threat has passed. Since most bacterial families in *C. elegans'* microbiome include beneficial as well as detrimental members (Samuel et al., 2016) that may smell similar to the worms, reversible transgenerational responses to multiple other bacterial species may have evolved as an adaptive strategy.

Our work provides proof of principle that transgenerational inheritance of learned avoidance is likely to benefit *C. elegans* in the wild and provides a pipeline for deciphering wild bacterial genomes to identify new small RNAs and characterize their roles in infected hosts. Although bacterial small molecules have been widely implicated in bacteria-host interactions (Bishai and Palm, 2021), the potential roles of bacterial small RNAs in regulating host physiology are largely understudied. Identifying different bacterial small RNAs that can interact with host organ systems will lead to a better understanding of this new dimension of bacteria-host interactions.

Acknowledgements

We thank Buck Samuel for sharing GRb0427 bacteria and the *C. elegans* Genetics Center for worm strains; W. Wang, J. Arly Volmar, and J. Miller (Genomics Core Facility, Princeton University); BioRender for model figure design software; the Murphy lab for discussion and feedback; and J. Ashraf and W. Keyes for assistance. C.T.M. is the Director of the Glenn Center for Aging Research at Princeton and the Lewis Sigler Institute for Integrative Genomics. This work was supported by a Pioneer Award to C.T.M. (NIGMS DP1GM119167), a Transformative R01 Award (1R01AT011963-01) to Z.G. & C.T.M., CDCP 75D30122C15113 to C.M., T32GM007388 (NIGMS) support of R.S.M., a Damon Runyon Fellowship (DRG-2481-22) to T.S., and an NSF predoctoral award to J.S..

Methods

Resource availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by Coleen T. Murphy (ctmurphy@princeton.edu).

Materials availability

Bacterial and *C. elegans* strains generated in this study are available on request to the lead contact.

Experimental model and subject details

Bacterial strains

The GRb0427 strain was a generous gift from Dr. Buck Samuel's lab. OP50 was obtained from the C.G.C. The PA14 strain was a gift from Prof. Zemer Gitai's lab. Control (L4440) and *maco-1* RNAi were obtained from the Ahringer RNAi library, and the respective sequences were verified. *P. mendocina* MSPm1 is part of the CeMbio (Dirksen et al., 2020) collection and was obtained from CGC.

Engineered bacterial strains

E. coli expressing the GRb0427 347nt intergenic region containing the 16-nucleotide match to *maco-1* was constructed by Gibson Assembly. The entire intergenic region was cloned out of GRb0427 and ligated into pZE27GFP using a double restriction digestion to open the plasmid and a single fragment Gibson assembly to ligate. This plasmid was then transformed into MG1655 *E. coli* using a standard transformation protocol. *E. coli* expressing the Pv1 small RNA was constructed in a similar method, using primers specific to the Pv1 region.

The deletion of the 16 nt region of Pv1 (that matches to *C. elegans maco-1*) from the GRb0427 genome was constructed by two-step allelic exchange using plasmid pEXG2. Briefly, ~600 bp fragments directly upstream and downstream of Pv1 sequence were amplified from GRb0427 gDNA using primer pairs (Pv1-KO1, Pv1-KO2) and (Pv1-KO3, Pv1-KO4), respectively. Overlap-extension PCR, with primer pair (Pv1-KO1, Pv1-KO4), was used to fuse together the upstream and downstream fragments. The final fragment was cloned into pEXG2, which was PCR linearized with primer pair (pEGX2-Lin1, pEGX2-seq2). The pEXG2 plasmid was integrated into GRb0427 by conjugation from donor strain *E. coli* s17 and exconjugants were selected on gentamycin 30 µg/mL and irgasan 100 µg/mL. Mutants of interest were counterselected on 15% sucrose and the proper deletion was confirmed via PCR, using primers (Pv1-seq1, Pv1-seq2), and sequencing of PCR products.

Primer details:
Pv1-KO1 - CGCACCCGTGGAAATTAATTGCTTCAGTGAAGGGCGG
Pv1-KO2 - TTCAGCATGCTTGCGGCTCGAGCACAGAAAGCAGATTAAATATGCGC
Pv1-KO3 - CTCGAGCCGCAAGCATGCTGAATTTTAGCCGTACCGAACAAGC
Pv1-KO4 - CCGGAAGCATAAATGTAAGCGTCCTTGTCGGGGC
pEGX2-Lin1 - CTTTACATTTATGCTTCCGGCTCGTA
pEGX2-Lin2 - AATTAATTTCCACGGGTGCGCATG
Pv1-seq1 - CGCACCCGTGGAAATTAATTGCTTCAGTGAAGGGCGG
Pv1-seq2 – CCGGAAGCATAAATGTAAGCGTCCTTGTCGGGGC

***C. elegans* strains**

The following strains were used in this paper and were obtained from the CGC: N2 (wild type), AU133: *agls17[Pmyo-2::mCherry + Pirg-1::gfp]*, FK181: *ksIs2 [Pdaf-7p::gfp + rol-6(su1006)]*, CQ759: *maco-1(ok3165)* 2X outcrossed, and JU1580 (wild type, natural *C. elegans* isolate).

Cultivation of bacterial strains

All bacterial strains were grown overnight at 37°C in a shaker (250 rpm). *E. coli* OP50, *P. vranovensis* GRb0427, *P. mendocina* MSPm1, and *P. aeruginosa* PA14 were grown in LB (10 g/l tryptone + 5 g/l yeast extract + 10 g/l NaCl in distilled water). *E. coli* expressing the Pv1 sRNA were grown in LB supplemented with 50 µg/mL Kanamycin. RNAi bacteria were grown in LB supplemented with 100 µg/ml carbenicillin and 12.5 µg/ml tetracycline.

General maintenance of *C. elegans* strains

Worm strains were maintained at 20°C on high-growth medium (HGM) plates (3 g/l NaCl, 20 g/l bacto-peptone, 30 g/l bacto-agar in distilled water, with 4 mL/L cholesterol (5 mg/mL in ethanol), 1 mL/L 1 M $CaCl_2$, 1 mL/L 1 M $MgSO_4$ and 25 mL/L 1 M KPO_4 buffer (pH 6.0) added to molten agar after autoclaving) on *E. coli* OP50.

Method details

Training plate preparation

Training plates were prepared by pipetting 800 uL of bacteria onto NGM (3 g/L NaCl, 2.5 g/L Bacto-peptone, 17 g/L Bactoagar in distilled water, with 1 mL/L cholesterol (5 mg/mL in ethanol), 1 mL/L 1M CaCl2, 1 mL/L 1M MgSO4, and 25 mL/L 1M potassium phosphate buffer (pH 6.0) added to molten agar after autoclaving) or HG plates. Pathogenic bacteria such as PA14 or GRb0427 were prepared on NGM plates to avoid overgrowth, while sRNA producing MG1655 were prepared on HG plates. For sRNA training, 200 µl of OP50 was spotted in the centre of a 6-cm NGM plate. Plates were stored at 25°C for 48hrs. RNAi bacteria were prepared on HG plates (supplemented with 1 mL/L 1M IPTG, and 1 mL/L 100 mg/mL carbenicillin) and kept at room temperature for 48 hrs.. Plates were then taken out of the incubator and allowed to cool to room temperature before moving animals onto them.

Bacterial choice assay plate preparation

Overnight bacterial cultures were diluted in LB to an $OD_{600} = 0.5$, and 25 µl of each bacterial suspension was spotted onto one side of a 60-mm NGM plate to make bacterial choice assay plates. These plates were incubated for 2 days at 25 °C.

Preparation of bacteria for small RNA isolation

GRb0427 and *E. coli* OP50 bacteria were cultured for 16 hours overnight. 1 ml of either bacterial culture was diluted to an OD = 0.5, plated on 100mm NGM plates and grown at 25 °C for 48 hours. For 15 °C GRb0427 small RNA isolation, overnight (37°C) cultures of GRb0427 bacteria were centrifuged for 10 min at 5,000*g*. The supernatant was removed, and the remaining pellet was resuspended in 5 ml of fresh LB. Washed bacteria were used to inoculate (1:500) fresh LB to grow at 15 °C for 36 hrs. 1 ml of this bacterial culture was then diluted to an OD = 0.5, plated on 100mm NGM plates and grown at 15 °C for 48 hours.

Bacterial lawns were collected from the surface of the plates using a cell scraper. 1 ml of M9 buffer was applied to the surface of the bacterial lawn, and the bacterial suspension obtained by scraping was transferred to a 15-ml conical tube. *E. coli* OP50 from 15 plates, or GRb0427 from 10 plates were pooled in each tube and pelleted at 4500*g* for 8 min.

The supernatant was discarded, and the bacterial pellet was resuspended in 900 uL of Trizol LS for every 100 µl of bacterial pellet. The pellet was resuspended by vortexing and the tubes containing the bacterial pellet were frozen at −80 °C.

Bacterial small RNA isolation

To isolate small RNA, bacterial pellets-Trizol suspensions were first incubated at 65 °C for 10 min with occasional vortexing. Debris were pelleted at 4500*g* for 5 min. The supernatant was transferred to 1.5 mL tubes (1 mL in each tube) and 200uL chloroform was added. Samples were mixed by inverting and centrifuged at 12,000*g* at 4 °C for 10 min. The aqueous phase obtained was used as input for small RNA extraction using the mirVana miRNA isolation kit. Extraction was done as per the manufacturer's instructions for small RNA (<200 nt) isolation. Purified small RNA was used immediately in aversive learning assays or for sequencing, or frozen at −80 °C until further use.

Training *C. elegans* on bacterial lawns and small RNAs

Wild-type N2 animals were synchronized by bleaching and grown until larval stage 4 (L4) on standard HG plates seeded with OP50. At L4 stage, they were transferred to training plates.

After 48 hrs at 25ºC the training plates were left on the bench top for 30 mins to allow them to reach room temperature. For sRNA training, 100 ug of sRNA was added to the OP50 spot on the sRNA training plates. For RNAi, 200 µL of 0.1M IPTG was spotted onto seeded RNAi plates and left to dry at room temperature before adding worms. Larval stage 4 (L4) worms were washed off plates using M9 and left to pellet on the bench top for 2-3 min. Then, 5 µl of worms were placed onto sRNA-spotted training plates, 10 µl onto OP50 plates, or 20 µl onto RNAi plates, *E. coli* expressing Pv1, GRb0427, or GRb0427Δ16 training plates. Worms were incubated on training plates at 20°C in separate containers for 24 hours. After 24 hours, worms were washed off plates using M9 and washed an additional 2-3 times to remove excess bacteria. Trained worms were tested in the aversive learning assay.

Aversive learning assay

On the day of the assay, bacterial choice assay plates were left at room temperature for 1 h before use. To start the assay, 1 µl of 1 M sodium azide was spotted onto each respective bacteria spot to be used as a paralyzing agent during choice assay. Worms were then washed off training plates in M9, allowed to pellet by gravity, and washed 2-3 additional times in M9. Using a wide orifice pipet tip, 5 µl of worms were spotted at the bottom of the assay plate, midway between the bacterial lawns. The assay plates were incubated at room temperature for 1 h. After that, the number of worms on each bacterial spot were counted. Plating a large number of worms (>200) on choice assay plates was avoided, because the worms clump at bacterial spots making it difficult to distinguish individual worms during counting, and also because high densities of worms can alter behavior.

For experiments testing behavior of the F1 generation, day 1 worms from parental (P_0) training were bleached and eggs were placed onto HG plates and left for 3 days at 20 °C. After 3 days, the F1 worms (Day 1 – 72 hours) were washed off HG plates with M9. Some of the pooled worms were subjected to the aversive learning assay, and the remaining worms were bleached to obtain eggs. The eggs were then placed onto HG plates, which were left at 20 °C. After 3 days the F2 progeny were tested, and the same steps were followed for subsequent progeny generations.

Annotation of the GRb0427 genome

The GRb0427 genome was downloaded from Burton et al., 2020 (ref) and run through an annotation pipeline in python. This pipeline searches for all open reading frames across the genome on both strands and makes a temporary gene list. It then filters genes by both size and overlap with other genes to give a final predicted gene list. Our pipeline predicted 4952 genes in the genome. Multiple genes were then selected and run through NCBI BLAST to confirm their identities.

After identifying the genes in the genome, we used Standalone BLAST, specifically, BLAST-nShort, to identify regions of homology between *maco-1* and the GRb0427 genome. This yielded 5 hits: 4 hits of 16 nucleotides and 1 of 20 nucleotides. Next, these hits were run through a python function to determine if they lie in an intergenic region, and this filtered our list down to a singular 16 nucleotide hit. This hit lies within a predicted 347nt intergenic region.

Bacterial small RNA sequencing

Prior to sRNA sequencing, each sample of GRb0427 sRNA was tested for *C. elegans* behavior. The size distribution of sRNA samples was examined on a Bioanalyzer 2100 using RNA 6000 Pico chip (Agilent Technologies). The sRNA sequencing protocol was similar to the protocol used in (Kaletsky et al., 2020). Briefly, around 300 ng of sRNA from each sample was first treated with RNA 5′ pyrophosphohydrolase (New England Biolabs) at 37 °C for 30 min, then converted to Illumina sequencing libraries using the PrepX RNA-seq library preparation protocol on the automated Apollo 324 NGS Library Prep System (Takara Bio). The treated RNA samples were ligated to two different adapters at each end, then reverse-transcribed to cDNA and amplified by PCR using different barcoded primers. The libraries were examined on Bioanalyzer DNA High Sensitivity chips (Agilent) for size distribution, quantified by Qubit fluorometer (Invitrogen), and then pooled at equal molar amount and sequenced on Illumina NovaSeq 6000 S Prime flowcell as single-end 122-nt reads. The pass-filter reads were used for further analysis.

Bacterial small RNA sequencing data analysis

4 replicates of GRb0427 small RNA and 3 replicates of *E. coli* OP50 small RNA were sequenced. Reads were mapped to the sequenced GRb0427 genome (Burton et al., 2020) using RNA STAR (Dobin et al., 2013). Default settings were used for the RNA STAR mapping. The resulting BAM files were then loaded into IGV genome browser (Thorvaldsdóttir et al., 2013) for analysis of the intergenic region. The peaks and read strands indicated that 3 small RNAs lie in our intergenic region of interest. Using the Sapphire promoter analysis software for *Pseudomonas* species (Coppens and Lavigne, 2020), we found highly confident predicted promoters that were consistent with the sequencing data. Of the 3 small RNAs one contains the 16 nt homology to *maco-1*, and this small RNA was named Pv1.

Determination of the operon context of Pv1

We examined if the Pv1 lies in a GRb0427 operon. Using the Operon Mapper operon detection software (Taboada et al., 2018), we found that while Pv1 is flanked by operons for iron metabolism and sugar transport, Pv1 itself is not part of an operon.

Pv1 structure prediction

The small RNA that has homology to *maco-1* was identified by our sequencing data to be 121 nucleotides in length. Using the mFold sRNA secondary structure prediction tool on

the UNAFold webserver (Zuker, 2003), we found that this RNA contains a long stem loop structure with the sequence match to *maco-1* beginning in a stem and ending after the turn of a loop. Interestingly P11's sequence match to *maco-1* has a similar secondary structure context.

Imaging and image analysis

daf-7p::gfp images of OP50, PA14, and GRb0427 worms were taken on a Nikon Eclipse Ti microscope. Worms were prepared and treated as described in 'Worm preparation for training'. Worms were mounted on 1% agar pads on glass slides and immobilized using 1 mM levamisole. *Z*-stack multi-channel (DIC and GFP) images of day-1 adult GFP-transgenic worms were acquired at 60X magnification. Maximum intensity projections of head neurons were built using Fiji. Quantification of mean fluorescent intensity was done using NIS-Elements software. Average pixel intensity was measured in each worm by drawing a Bezier outline of the neuron cell body for 2 ASI head neurons.

For *irg-1p::gfp* quantification, worms were prepared as described in 'Worm preparation for training' and imaged at 20× magnification on a Nikon A1 R confocal microscope. Image analysis was done with Fiji, where ROIs were drawn around each animal in the field of vision and mean intensity values for all regions of interest were recorded and plotted.

Survival assay

Survival assay on GRb0427 and GRb0427Δ16 lawns:

GRb0427 and GRb0427Δ16 bacteria were grown in liquid culture overnight (37°C) and diluted 1:4 to an OD = 0.5. 750 µL of diluted GRb0427 or GRb0427Δ16 was spread to completely cover six 6-cm NGM plates for each bacterial genotype. Plates were incubated for 2 days at 25°C to allow bacterial growth. Plates were equilibrated to 20°C before adding worms (84 hours post-bleach) to plates. Survival assays were performed at 20°C. The assay plates were counted every 6-9 h. Every 48h, worms were moved onto new plates.

Survival assay on 25ºC-grown and 15ºC-grown GRb0427 lawns:

Preparation of 25ºC-grown GRb0427 survival assay plates - Overnight (37°C) cultures of GRb0427 bacteria were diluted in LB to (OD_{600}) = 0.5 and used to fully cover six 6-cm nematode growth medium (NGM) plates. The plates were incubated for 2 days at 25°C.

Preparation of 15ºC-grown GRb0427 survival assay plates - Overnight (37°C) cultures of GRb0427 bacteria were centrifuged for 10 min at 5,000g. The supernatant was removed, and the remaining pellet was resuspended in 5 ml of fresh LB. Washed bacteria were used to inoculate (1:500) fresh LB to grow at 15 °C for 36 hrs. Cultures were diluted in LB to an OD_{600} = 0.5 and used to seed NGM plates. The plates were incubated at 15 °C for 2 days.

Assay procedure - Plates were equilibrated to 20°C before adding Day 1 (72 hours post-bleach) worms to plates. Survival assays were performed at 20°C. The assay plates were counted every 6-9 h. Every 24h, worms were moved onto new plates of 25ºC-grown and 15ºC-grown GRb0427 (prepared as described above).

PCR detection of the Pv1 small RNA

Total RNA and small RNA were extracted

from 25°C-grown and 15°C-grown GRb0427 using the mirVana miRNA isolation kit (see **Key Resources Table**), and reverse-transcribed to DNA (using SuperScript III; see **Key Resources Table**). A 62 bp region and an 88 bp region of Pv1 were identified using the following PCR primers:

Pv1(62bp)Fwd:
CTGTGACGATTACAAATTAAC

Pv1(62 bp)Rev: GCCGTACCGAACAAG

Pv1(88 bp)Fwd: GCCTAGCACTGGTTAG

Pv1(88bp)Rev:
CTGTGACGATTACAAATTAAC

Statistical analysis

Survival assays were assessed using Log-rank (Mantel-Cox) tests. For the comparison of choice indices between more than two genotypes, one-way ANOVA with Tukey's multiple comparisons test were used. For comparisons of choice indices between genotypes and between conditions (naïve vs learned), two-way ANOVA with Tukey's multiple comparisons test were used. Unpaired t tests were performed for comparisons between two groups. Experiments were repeated on separate days with separate populations, to confirm that results were reproducible. Prism 9 software was used for all statistical analyses.

Supplemental figures:

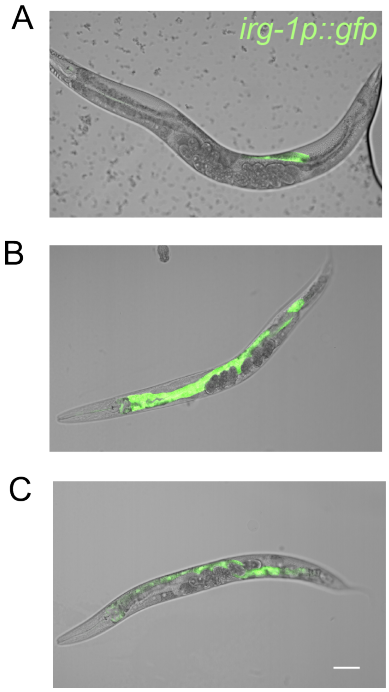

Figure S1. Expression of an *irg-1p::gfp* reporter in representative OP50-trained (A), PA14-trained (B) and GRb0427-trained (C) animals. A-C are merged confocal micrographs of brightfield and GFP channels. Scale bar = 40 μm.

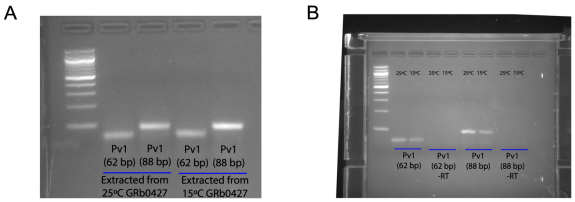

Figure S2. (A) 62 bp and 88 bp of Pv1 amplified by two different primer sets from total RNA pool extracted from 25°C and 15°C-grown GRb0427 (indicating expression of the Pv1 small RNA under both temperature conditions). (B) 62 bp and 88 bp of Pv1 amplified by the same primer sets as in (A) (also see STAR Methods) from small RNA pool extracted from 25°C and 15°C-grown GRb0427. +RT and -RT indicate presence and absence respectively of the reverse transcriptase enzyme.

Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
P. vranovensis GRb0427	Buck Samuel	
E. coli OP50	CGC	
Pseudomonas aeruginosa PA14	Z. Gitai	
Pseudomonas mendocina MSPm1	CGC (CeMbio)	
E. coli MG1655	Z. Gitai	
E. coli expressing GFP	This paper	
E. coli expressing Pv1	This paper	
GRb0427 Δ16 nt	This paper	
E. coli S17	Z. Gitai	
Chemicals, peptides, and recombinant proteins		
Sodium azide	Fisher Scientific	S2271-25
Potassium Hydroxide	Fisher Scientific	P258-212
Sodium Hypochlorite	Thermo Fisher Scientific	SS290-1
Peptone	Fisher Scientific	DF0118-07-2
Sodium Chloride	Sigma-Aldrich	S9888
Bacto-Agar	Fisher Scientific	DF0140-07-4
Potassium Phosphate monobasic	Fisher Scientific	7778-77-0
Potassium Phosphate dibasic	Millipore Sigma	7758-11-4
Magnesium Sulfate Anhydrous	Fisher Scientific	7487-88-9
Calcium Chloride	Fisher Scientific	AC349615000
Yeast Extract	Fisher Scientific	DF0127-17-9
Tryptone	Biosciences	211705
Sodium Phosphate Dibasic Anhydrous	Fisher Scientific	7558-79-4
Cholesterol	Fisher Scientific	C314-500
Carbenicillin	Sigma-Aldrich	C4316
Tetracycline	Sigma-Aldrich	87128
Kanamycin	Fisher BioReagents	25389-94-0
Trizol LS Reagent	Fisher Scientific	10-296-010
Levamisole solution	Vector Laboratories	SP-500
Chloroform	Sigma-Aldrich	319988
IPTG	Fisher bioreagents	367-93-1
Critical Commercial Assays		
mirVana, miRNA Isolation Kit with phenol	Thermo Fisher Scientific	AM1560
Superscript III First Synthesis System	Invitrogen	18080-051
Experimental Models: Organisms/Strains		
C. elegans: Strain N2 var. Bristol: wild-type	*Caenorhabditis* Genetics Center	WB Strain: N2
C. elegans: Strain *ksIs2 [daf-7p::GFP + rol-6(su1006)]*	*Caenorhabditis* Genetics Center	WB Strain: FK181

C. elegans: Strain JU1580	*Caenorhabditis* Genetics Center	WB Strain: JU1580
C. elegans: Strain *maco-1(ok3165)* 2X outcrossed	This paper	WB Strain: CQ759
C. elegans: Strain *Pmyo-2::mCherry + Pirg-1::GFP*	*Caenorhabditis* Genetics Center	WB Strain: AU133
Oligonucleotides		
Pv1-KO1 - CGCACCCGTGGAAATTAATTGCTTCAGTGAAGGGCGG	This Study	N/A
Pv1-KO2 - TTCAGCATGCTTGCGGCTCGAGCACAGAAAGCAGATTAAATATGCGC	This Study	N/A
Pv1-KO3 - CTCGAGCCGCAAGCATGCTGAATTTTAGCCGTACCGAACAAGC	This Study	N/A
Pv1-KO4 - CCGGAAGCATAAATGTAAGCGTCCTTGTCGGGGC	This Study	N/A
pEGX2-Lin1 - CTTTACATTTATGCTTCCGGCTCGTA	This Study	N/A
pEGX2-Lin2 - AATTAATTTCCACGGGTGCGCATG	This Study	N/A
Pv1-seq1 - CGCACCCGTGGAAATTAATTGCTTCAGTGAAGGGCGG	This Study	N/A
Pv1-seq2 – CCGGAAGCATAAATGTAAGCGTCCTTGTCGGGGC	This Study	N/A
ok3165 Fwd – CTGTTTCTGTGGAACTGG	This Study	
ok3165 Rev – CAGAGTTTGTGTTTAAATTATTCC	This Study	
ok3165 WT rev – GGAGGAAAACGATATTGACG	This Study	
Pv1 (62 bp) Fwd: CTGTGACGATTACAAATTAAC	This Study	
Pv1 (62 bp) Rev: GCCGTACCGAACAAG	This Study	
Pv1 (88 bp) Fwd: GCCTAGCACTGGTTAG	This Study	
Pv1 (88 bp) Rev: CTGTGACGATTACAAATTAAC	This Study	
	This Study	
Recombinant DNA		

Plasmid: pL4440 RNAi control	*C. elegans* RNAi Collection (Ahringer)	
Plasmid: pL4440-*maco-1* RNAi	*C. elegans* RNAi Collection (Ahringer)	
Plasmid: pBAD18-Amp	Kaletsky et al., 2020	
Plasmid: PBAD18-Amp-P11	Kaletsky et al., 2020	
Plasmid: pZE27GFP	Addgene #75452	
Plasmid: pZE27-intergenic	This paper	
Plasmid: pZE27-Pv1	This paper	
Software and Algorithms		
Prism 8	GraphPad Prism	https://www.graphpad.com/scientific-software/prism/
NIS-Elements AR	Nikon Instruments	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research
Standalone BLAST	NCBI, NIH	https://www.ncbi.nlm.nih.gov/books/NBK52637/
RNA STAR	https://code.google.com/archive/p/rna-star/	
Integrative Genomics Viewer	https://software.broadinstitute.org/software/igv/	
SAPPHIRE	https://sapphire.biw.kuleuven.be/	
Operon Mapper	https://biocomputo.ibt.unam.mx/operon_mapper/	
mFold	The UNAFold Web Server	http://www.unafold.org/mfold/applications/rna-folding-form.php
Fiji	https://imagej.net/software/fiji/	

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