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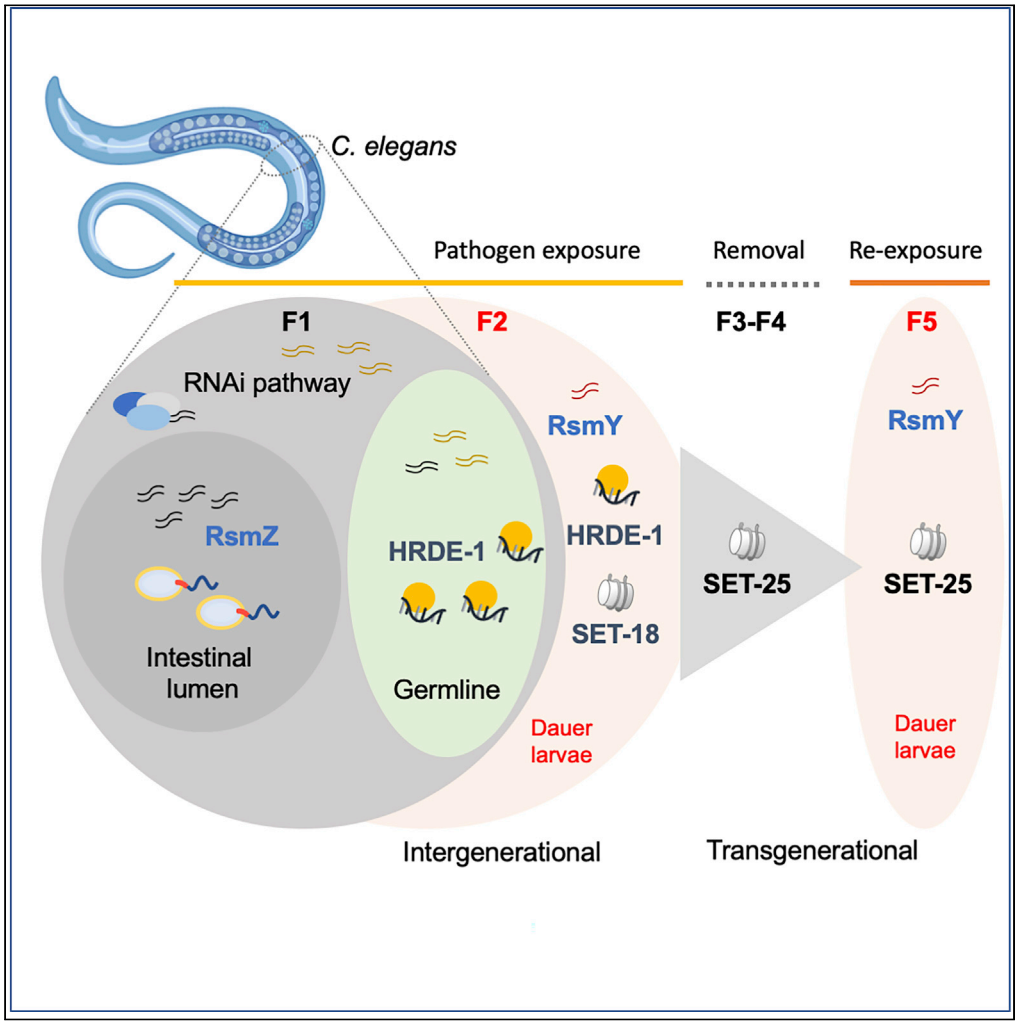

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Highlights

sRNAs of *P. aeruginosa* are required for an intergenerational behavior in *C. elegans*

Nuclear RNAi in the *germline* is required for the intergenerational inheritance of PIDF

Host HMT is required *for* maintenance of the transgenerational memory

Interspecies sRNA *expression* through generations of interaction is dynamic

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Interspecies effectors of a transgenerational memory of bacterial infection in *Caenorhabditis elegans*

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SUMMARY

The inheritance of memory is an adaptive trait. Microbes challenge the immunity of organisms and trigger behavioral adaptations that can be inherited, but how bacteria produce inheritance of a trait is unknown. We use *Caenorhabditis elegans* and its bacteria to study the transgenerational RNA dynamics of interspecies crosstalk leading to a heritable behavior. A heritable response of *C. elegans* to microbes is the pathogen-induced diapause (PIDF), a state of suspended animation to evade infection. We identify RsmY, a small RNA involved in quorum sensing in *Pseudomonas aeruginosa* as a trigger of PIDF. The histone methyltransferase (HMT) SET-18/SMYD3 and the argonaute HRDE-1, which promotes multi-generational silencing in the germline, are also needed for PIDF initiation. The HMT SET-25/EHMT2 is necessary for memory maintenance in the transgenerational lineage. Our work is a starting point to understanding microbiome-induced inheritance of acquired traits, and the transgenerational influence of microbes in health and disease.

INTRODUCTION

Microbes vastly influence life history traits of their host organisms. Some of these traits are behavioral strategies to survive bacterial pathogenesis that are inherited to the progenies. Animals and microbes interact persistently, and their relationship has played a crucial role in evolution (Alegado and King, 2014; Provorov et al., 2008).

Bacterivore nematodes are among the most ancient and abundant metazoans in the biosphere (Poinar, 2015; Cobb, 1914; van den Hoogen et al., 2019; Samuel et al., 2016). The nematode *Caenorhabditis elegans* feeds on a great variety of bacteria from commensals to human pathogens. The interaction between bacteria and *C. elegans* can be studied as a whole system—a *holobiont* and offers an excellent frame to gain insight into long-term interspecies relationships, and bidirectional phenotypic modulation (Celluzzi and Masotti, 2016; Legüe and Calixto, 2019).

C. elegans manifests both transient and long-term adaptive behaviors to detrimental bacterial diets. For instance, worms identify pathogens based on their smell and learn to avoid them (Zhang et al., 2005). In the host, pathogens can induce adaptive heritable strategies to ensure the survival of progenies (Palominos et al., 2017; Kaletsky et al., 2020). Attractive or repulsive odors can also generate long-lasting heritable memory (Remy, 2010; Moore et al., 2019; Pereira et al., 2020).

C. elegans enters diapause—a period of suspended development—after two generations feeding on pathogens of moderate virulence such as *Pseudomonas aeruginosa* PAO1 and *Salmonella enterica* (Palominos et al., 2017). This pathogen-induced diapause formation (PIDF) is the entry in an alternate developmental state called dauer larvae. Dauers undergo drastic morphological changes such as the closure of their mouth. This allows exclusion of pathogenic microbes and prevents infection (Palominos et al., 2017; Gabaldón et al., 2020). PIDF requires infection with live bacteria and is dependent on effectors of the host RNAi (RNAi) machinery, including dsRNA transporters involved in systemic RNAi expressed in the intestine (SID-2) and in all non-neuronal tissues (SID-1). These requirements allowed us to hypothesize that small RNAs (sRNAs) from intestinal bacteria could be needed to trigger PIDF. The establishment of PIDF requires the maternal germline and bacterial colonization for two generations, suggesting an intergenerational transmission of information. However, in animals whose ancestors were infected, PIDF is triggered in the first generation after exposure to pathogens, indicating that pathogenic memory is inherited

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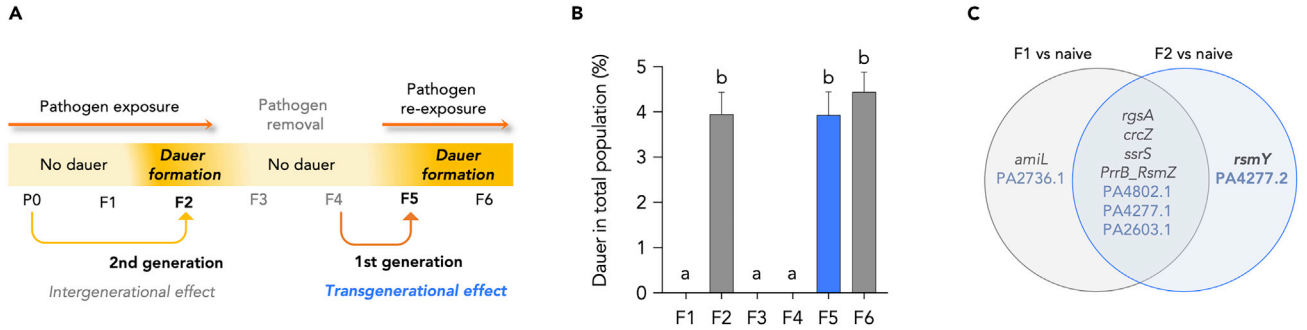

Figure 1. Interaction with pathogenic bacteria triggers a transgenerational defensive response in *C. elegans*

(A) Schematic representation of the transgenerational paradigm of pathogen-induced dauer formation (PIDF).

(B) Quantification of dauer formation for six generations in the transgenerational PIDF paradigm shown in A. Data are represented as mean ± SEM. a and b denote conditions that are significantly different from each other. Conditions with the same letter are not significantly different.

(C) Diagram showing the specific expression of *P. aeruginosa* ncRNAs in the intestine of *C. elegans* in the first and the second generation exposed to pathogens. Details of statistical analysis can be found in Data S1.

transgenerationally (Palominos et al., 2017). How these memories are established and what are the bacterial triggers are not well understood.

sRNAs can influence multiple generations, either by amplification and maintenance of heritable sRNAs (Houri-Zeevi and Rechavi, 2017) or indirectly, by sRNA-mediated epigenetic modifications (Lev et al., 2017; Kalinava et al., 2017). Engineered double-stranded sRNAs (dsRNAs) can be transferred between generations in *C. elegans* (Marré et al., 2016) and moved from distant tissues to the germline (Jose, 2015; Devanapally et al., 2015; Posner et al., 2019). A question that emerges is whether interspecies sRNAs can modulate life history traits in the hosts that last for generations. In acute host-pathogen interactions, bacterial noncoding RNAs can modulate both bacterial virulence and impact transcription in the host (Westermann et al., 2016; Koeppen et al., 2016; Low et al., 2018; Betin et al., 2019). Host sRNAs can also affect bacteria (Liu et al., 2016). A bacterial sRNA influences pathogen avoidance in *C. elegans* (Kaletsky et al., 2020), suggesting this can be one of the mechanisms by which bacteria impact host behavior. We sought to identify the bacterial triggers of PIDF. In this work, we identified that RsmY, a *P. aeruginosa* sRNA involved in quorum sensing as a mediator of the defensive behavior in the nematode. RsmY and its homolog RsmZ are noncoding RNAs of 124 and 116 nucleotides, respectively, regulated by the GacS/A two-component system (Kay et al., 2006; Brencic et al., 2009). They act by sequestering the inhibitory protein RsmA that regulates a myriad of metabolic processes in *P. aeruginosa*. RsmY and RsmZ, however, have different contributions to PIDF when colonizing *C. elegans*. While RsmY mutation impairs PIDF, RsmZ is partially needed for wild-type dauer formation. This suggests that their role in PIDF is not fully explained by RsmA action. Furthermore, we studied a germline argonaute and histone modifications that could mediate transgenerational PIDF memory. We show that SET-18/SMYD3 and HRDE-1 are needed for PIDF to take place. Importantly, SET-25/EHMT2 is necessary for maintaining the transgenerational memory of pathogen encounters.

In this study, we also dissect the sRNA dynamics in naive bacteria and the holobiont throughout their bacteria-host relationship for six generations in a transgenerational paradigm. In this paradigm, animals are fed pathogenic bacteria interrupted by two generations feeding on non-pathogens. We identified the cohorts of sRNAs from pathogenic and non-pathogenic bacteria in naive and intestinal states. We found that specific bacterial sRNAs change their expression levels between nematode generations. Gene expression also evolves in the worm over each generation, suggesting an intimate reciprocal interaction between bacteria and host. Altogether, our findings provide insights into the bacteria-host dynamics across generations, by correlating global sRNA-transcriptomics with an adaptive behavior in *C. elegans*.

RESULTS

Bacterial sRNA expression depends on the host previous exposure to same bacteria

Pathogenic microbes induce a transgenerational defensive strategy in *C. elegans* upon persistent intestinal infection (Figures 1A and 1B and Palominos et al., 2017). In contrast to diapause formation induced by

Table 1. Bacterial sRNAs genes are differentially expressed in the intestines of *C. elegans* F1 and F2 generations compared to naive bacteria

F1					F2				
Gene name	Biotype	Genomic context	log2FC	padj	Gene name	Biotype	Genomic context	log2FC	padj
rgsA	ncRNA	overlapped	5,1	1,E−05	***rgsA***	ncRNA	overlapped	6,6	8,E−04
ssrS	ncRNA	Nested	3,0	6,E−03	***ssrS***	ncRNA	nested	4,5	9,E−05
crcZ	sRNA	nested overlapped	3,2	5,E−03	***crcZ***	ncRNA	nested	4,3	1,E−04
amiL	sRNA	nested overlapped	4,2	2,E−03	*rsmY**	ncRNA	nested	4,1	1,E−04
rsmZ	sRNA	nested overlapped	4,9	5,E−06	***rsmZ***	sRNA	nested	6,9	4,E−08
PA2736.1	tRNA	nested overlapped	6,4	2,E−03	PA4277.2*	tRNA	nested	4,1	6,E−03
PA4802.1	tRNA	Nested	4,3	7,2E−05	**PA4802.1**	tRNA	nested	5,0	1,E−05
PA4277.1	tRNA	Nested	4,0	1,E−04	**PA4277.1**	tRNA	nested	4,1	1,E−04
PA2603.1	tRNA	Nested	2,9	5,E−03	**PA2603.1**	tRNA	nested	3,5	8,E−04

Genes in bold are overexpressed in F1 and F2. Genes with asterisks are only differentially expressed in the F2.

starvation or crowding, PIDF is RNAi-dependent and is elicited in the second generation of animals exposed to pathogens. The dependence on RNAi and the transgenerational nature of PIDF point at sRNAs as initiators of this response. We studied the transcriptional changes in sRNAs in intestinal bacteria in the first two generations of synchronized young larvae (L2), the stage that precedes dauer formation. To identify sRNAs triggers of PIDF inheritance, we focused on bacterial sRNAs that are specifically overexpressed in the second generation of infected animals. We excluded transcriptional changes in bacteria that reflect adaptation to new carbon sources or are needed for infection to occur, mainly in the first generation. We performed sRNA-seq of naive bacteria (cultured in LB and unexposed to host) and dual-RNAseq of F1 and F2 nematodes, 24 h from birth after feeding naive bacteria of the same culture time (Figure S1). Certain bacterial sRNAs meaningful for interspecies communication such as intergenic, *cis*-encoded, and sRNA fragments could be underreported (Diallo and Provost, 2020; Subramanian et al., 2019; Liu et al., 2018). To overcome this, we complemented the traditional database annotation with the unbiased strategy of finding transcriptional peaks (TPs) and using their genomic coordinates to define a genomic feature (Gabaldón et al., 2020); and STAR Methods). We classified them as *known* sRNAs; *partially novel*; and *novel*, located in intergenic regions (Figure S2). Raw gene expression data were normalized using the *Trimmed Mean of M-values* (TMM) method, which allows us to estimate relative RNA production levels from different abundances, lengths, and compositions (Robinson and Oshlack, 2010).

Differential expression of bacterial sRNAs was done comparing the transcriptomes of naive with intestinal bacteria. This experiment allowed us to discriminate gene expression in bacteria in experienced F2 versus inexperienced F1 hosts. Bacterial ncRNAs differentially expressed in F1 and F2 are shown in Table 1 (Full list of TPs and their genomic contexts is shown in Data S1, mean dispersion in Figure S3). Two ncRNA transcripts, the sRNAs RsmY and the tRNA-Gly PA4277.2, were specifically upregulated in the second generation of animals (Figure 1C). This suggests that the expression of these bacterial genes is regulated as a consequence of a previous encounter between bacteria and nematodes. RsmZ, an sRNA that shares the GacS/GacA pathway with RsmY (Valverde et al., 2003), is overexpressed stably since the first generation interacting with *C. elegans*. Both RsmY and RsmZ are well characterized bacterial regulatory RNAs involved in biofilm formation, type VI secretion system, and quorum sensing (Kay et al., 2006; Rutherford and Bassler, 2012). The dynamic expression of RsmY and RsmZ could have a role in interspecies communication leading to behavioral adaptations in the host.

Quorum sensing RsmY sRNA is required for PIDF defensive response

We tested the role of Rsm sRNAs in PIDF by using single and double mutant bacteria of *rsm* genes (Kay et al., 2006; Heurlier et al., 2004). RsmY or RsmZ acts additively and/or redundantly, so single mutations of either do not affect virulence traits such as biofilm formation and exopolysaccharide production

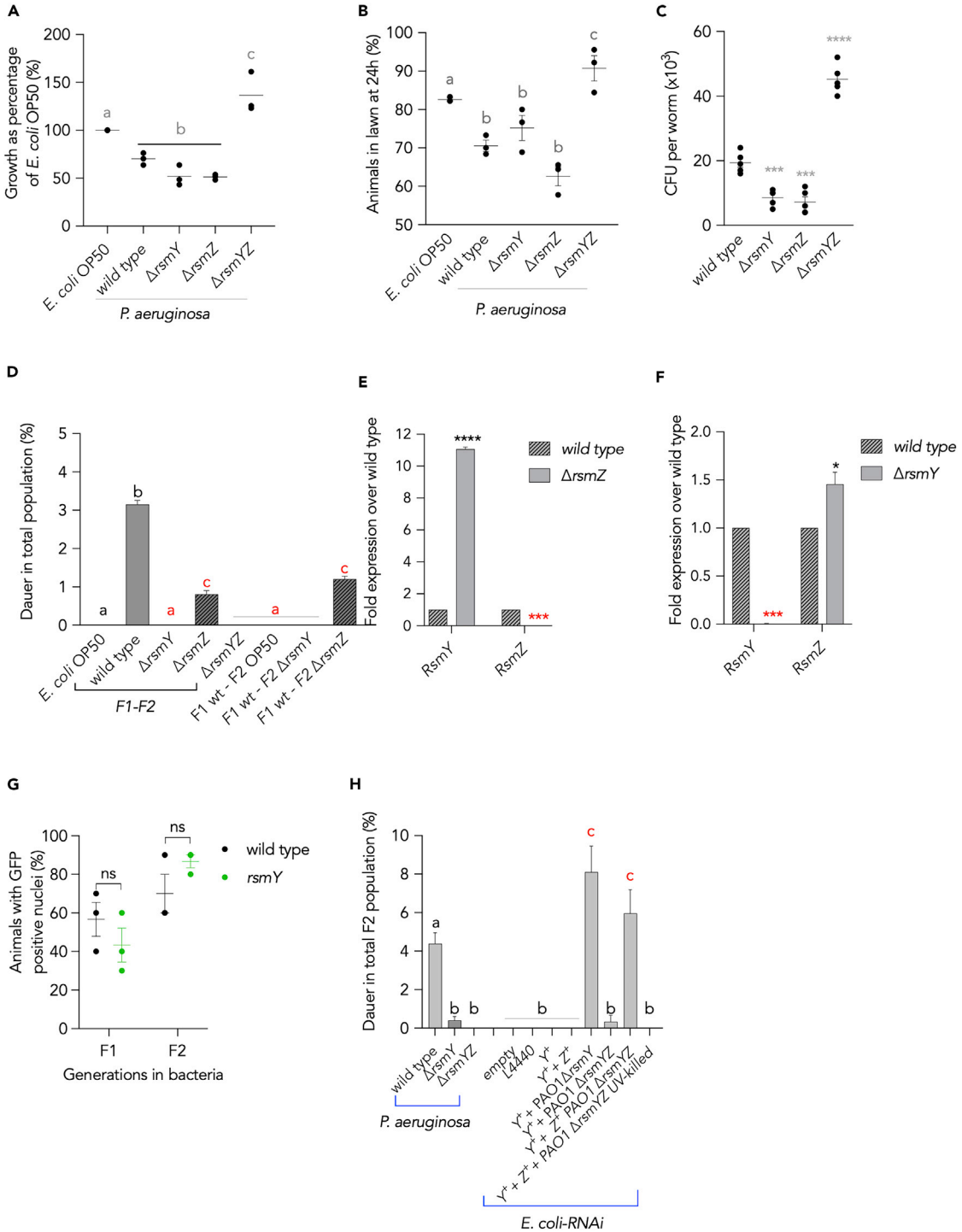

Figure 2. Intestinal *P. aeruginosa* sRNA RsmY is required for PIDF initiation in *C. elegans*

(A) Growth of *C. elegans* on *rsm* mutants as percentage of growth on *E. coli* OP50.

(B) Percentage of animals that remain in lawns of wild type, single, and double *rsm* mutants.

(C) Colony-forming units (CFU) in *C. elegans* intestine of wild type, single, and double *rsm* mutants.

(D) PIDF quantification for two generations on wild type and *rsm* mutants.

Figure 2. *Continued*

(E and F) Quantification of RsmY and RsmZ RNA expression in Δ*rsmY* and Δ*rsmZ* mutant bacteria.

(G) Percentage of animals with DAF-16:GFP positive nuclei.

(H) Dauer formation in animals feeding on bacteria expressing RsmY and/or RsmZ heterologously. Data are represented as mean ± SEM. In A, B, D, and H, a, b, and c denote conditions that are significantly different from each other. Conditions with the same letter are not significantly different. p-value *<0.0332, **<0.0021, ***<0.0002, ****<0.0001.

in vitro (Bordi et al., 2010) or extracellular products such as hydrogen cyanide, 2,4-diacetylphloroglucinol, or exoproteases (Valverde et al., 2003). We asked whether *rsm* mutations retain their pathogenicity *in vivo* in *C. elegans* compared to wild-type *P. aeruginosa* PAO1. We measured brood size, avoidance behavior, and intestinal colonization. *C. elegans* population size on Δ*rsmY* or Δ*rsmZ* was similar to those grown on wild-type *P. aeruginosa* PAO1, while growth in the double mutant Δ*rsmYZ* was larger (Figure 2A). Wild-type *P. aeruginosa* PAO1 induces lawn avoidance in nematodes 24 after the first exposure (Figure 2B). Avoidance of Δ*rsmY* and Δ*rsmZ* lawns was similar to wild-type bacteria. Δ*rsmYZ*, however, did not induce avoidance (Figure 2B). These results show that single *rsm* mutations do not affect the fitness of pathogens *in vivo* while loss of both genes eliminates virulence. We quantified the growth of each mutant in the *C. elegans* intestine by counting colony-forming units (CFU). Δ*rsmY* and Δ*rsmZ* formed over 10^3 individual bacteria per worm intestine, a large but significantly smaller number than wild-type bacteria (Figure 2C). Contrary to single mutants, Δ*rsmYZ* bacteria were many more in number than wild type *in vivo* (Figure 2C). This suggests that virulence is not correlated with a greater number of colonies in the intestine, and that the dynamics of organization inside the intestinal lumen may be more relevant than colony count for the bacteria-worm communication.

We grew *C. elegans* in *rsm* lawns for two generations to measure PIDF. Δ*rsmY* failed to induce diapause in the F2, while Δ*rsmZ* induced a smaller percentage than wild type (Figure 2D). As expected, avirulent Δ*rsmYZ* did not induce PIDF. To address the requirement of RsmY specifically in the F2, embryos from F1 hermaphrodites grown on wild-type *P. aeruginosa* were switched to Δ*rsmY* or Δ*rsmZ* in the second generation. Hypochlorite treatment to obtain embryos between generations eliminates previous bacteria. The change from wild-type *P. aeruginosa* to Δ*rsmY* abolished PIDF (Figure 2D) while the change to Δ*rsmZ* induced fewer dauers than wild type. These results show that RsmY is necessary for defensive diapause entry in *C. elegans,* with a key role in the F2. RsmZ, although not indispensable for PIDF, is required for the wild-type response. Δ*rsmY* mutant expressed similar levels of RsmZ than wild-type *P. aeruginosa* while Δ*rsmZ* bacteria show over a 10-fold increase in RsmY (Figures 2E and 2F), consistent with RsmZ being a repressor of the RsmY promoter (Kay et al., 2006). This result also shows that RsmZ cannot compensate for the loss of RsmY in triggering PIDF. Taken together, these results show that loss of *rsmY* but not *rsmZ* eliminates defensive diapause in the host.

We previously showed that *P. aeruginosa* PAO1 induces nuclear localization of DAF-16 as part of the defensive response of animals (Palominos et al., 2017). To further clarify whether RsmY loss affects the response of *C. elegans* to pathogens, we quantified the nuclear expression of DAF-16 in mutant bacteria compared to wild-type *P. aeruginosa*. Figure 2G shows that RsmY and wild-type *P. aeruginosa* are indistinguishable in inducing DAF-16 translocation. These results further support that RsmY loss does not affect the pathogenic potential of *P. aeruginosa in vivo*.

To test whether *rsmY* RNA alone could induce diapause or it required other bacterial factors, we inserted the RsmY or RsmZ sequences into a vector for double-stranded RNA expression (Fire et al., 1998). In an *Escherichia coli* OP50 modified to produce dsRNA (Xiao et al., 2015), we expressed RsmY (herein *E. coli-rsmY+*) or RsmZ (*E. coli-rsmZ+*). *E. coli-rsmY* + or *E. coli-rsmZ+* was induced to produce dsRNA and fed to nematodes. *E. coli-rsmY* + or the mix with *E. coli-rsmZ+* was unable to trigger PIDF, suggesting that other pathogen's components are also required for the elicitation of behavior (Figure 2H, growth is shown in Figure S4). Because *ΔrsmY* and *ΔrsmYZ* bacteria did not trigger PIDF, we fed animals with mixes of mutant *ΔrsmY* or *ΔrsmYZ* with *E. coli-rsmY+* and/or *E. coli-rsmZ+* (Figure 2H). The supplementation of *ΔrsmY* with *E. coli-rsmY* + triggered the production of large numbers of dauers in the second generation. In contrast, *ΔrsmYZ* mixed with *E. coli-rsmY* + did not (Figure 2H), suggesting *rsmZ* is also needed. *ΔrsmYZ* supplemented with *E. coli-rsmY+* and *E. coli-rsmZ* + produced PIDF, an effect that is abolished when *P. aeruginosa* is UV-killed. This result shows that RsmY expression in *E. coli* is sufficient for dauer induction in the presence of a pathogenic background with intact RsmZ. This suggests that RsmZ is needed for pathogen recognition highlighting a different but complementary role for each sRNA in triggering PIDF.

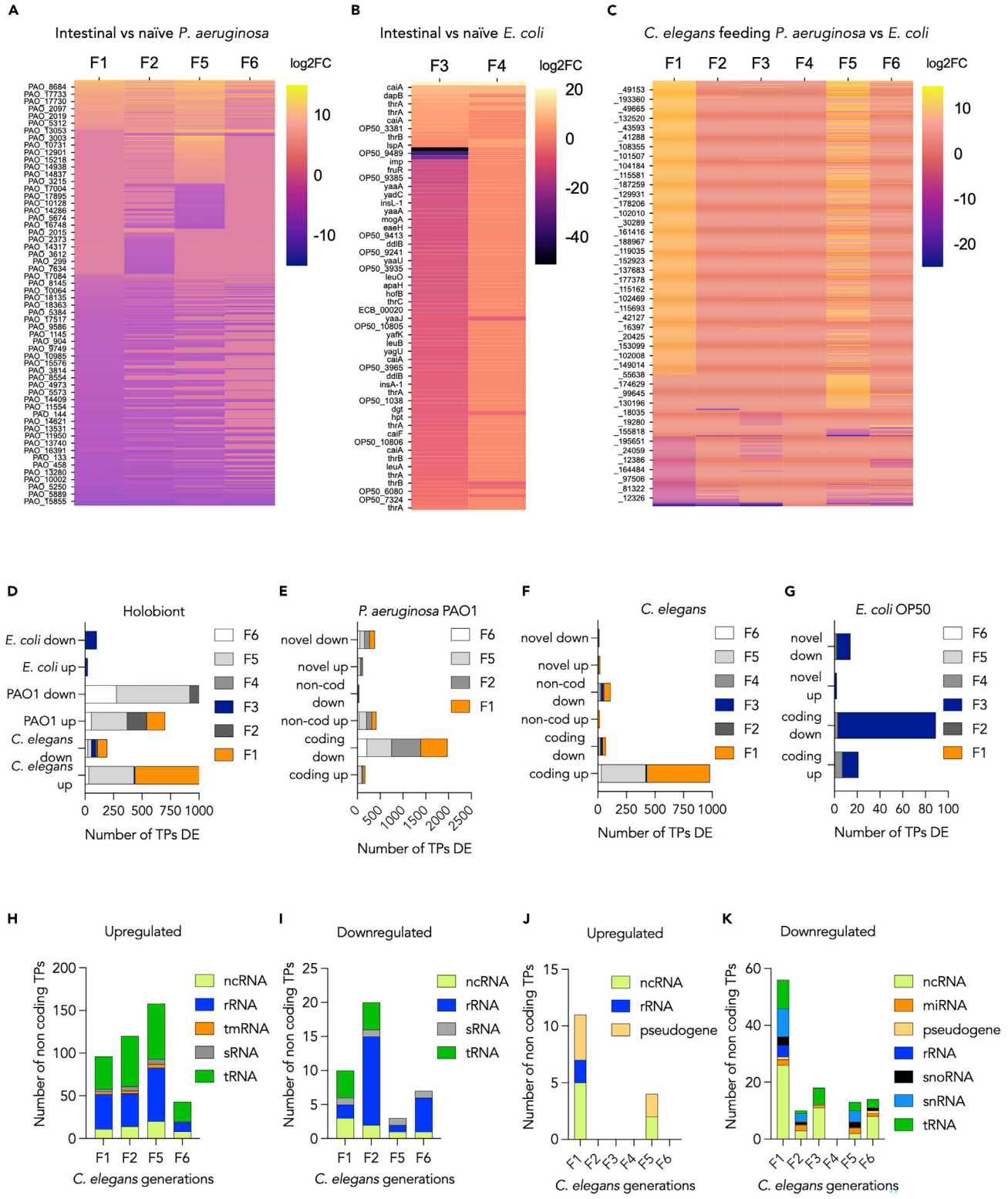
A
Intestinal vs naïve P. aeruginosa
F1 F2 F5 F6
log2FC
10
0
-10
B
Intestinal vs naïve E. coli
F3 F4
log2FC
20
0
-20
-40
C
C. elegans feeding P. aeruginosa vs E. coli
F1 F2 F3 F4 F5 F6
log2FC
10
0
-10
-20
D
Holobiont
E. coli down
E. coli up
PAO1 down
PAO1 up
C. elegans down
C. elegans up
F6 F5 F4 F3 F2 F1
0 250 500 750 1000
Number of TPs DE
E
P. aeruginosa PAO1
novel down
novel up
non-cod down
non-cod up
coding down
coding up
F6 F5 F2 F1
0 500 1000 1500 2000 2500
Number of TPs DE
F
C. elegans
novel down
novel up
non-cod down
non-cod up
coding down
coding up
F6 F5 F4 F3 F2 F1
0 250 500 750 1000
Number of TPs DE
G
E. coli OP50
novel down
novel up
coding down
coding up
F6 F5 F4 F3 F2 F1
0 20 40 60 80 100
Number of TPs DE
H
Upregulated
Number of non coding TPs
0 50 100 150 200
ncRNA
rRNA
tmRNA
sRNA
tRNA
F1 F2 F5 F6
C. elegans generations
I
Downregulated
Number of non coding TPs
0 5 10 15 20 25
ncRNA
rRNA
sRNA
tRNA
F1 F2 F5 F6
C. elegans generations
J
Upregulated
Number of non coding TPs
0 5 10 15
ncRNA
rRNA
pseudogene
F1 F2 F3 F4 F5 F6
C. elegans generations
K
Downregulated
Number of non coding TPs
0 20 40 60
ncRNA
miRNA
pseudogene
rRNA
snoRNA
snRNA
tRNA
F1 F2 F3 F4 F5 F6
C. elegans generations

Figure 3. Dynamic sRNA expression changes in the holobiont throughout the generations in the transgenerational paradigm
(A–C) TPs that change in expression in at least one generation in *P. aeruginosa* (A), *E. coli* OP50, (B) or *C. elegans* (C).
(D) Number of TPs differentially expressed in the holobiont (*C. elegans- P. aeruginosa* or *C. elegans- E. coli*) on each generation of interaction.
(E–G) Number of TPs that are matching, nested, or overlapped in coding and noncoding genes in *P. aeruginosa* (E), *C. elegans* (F) and *E. coli* (G).
(H–K) Number of TPs nested or matching noncoding genes of intestinal *P. aeruginosa* (H and I) and *C. elegans* (J and K) per sRNA biotype per generation, upregulated (H and J) or downregulated (I and K).

RsmY and RsmZ require live *Pseudomonas* to cause PIDF and could promote dauer formation by targeting gene expression of bacteria or *C. elegans*. We examined *in silico* the existence of perfectly matching sequences to RsmY and RsmZ in the *C. elegans* genome using the BLAST + tool (Camacho et al., 2009). Our criteria for *bona fide* interaction were an E-value <1. A number of hits, located in coding and noncoding regions (Table S1), did not meet the stringency parameters (see STAR Methods). Our results suggest that if RsmY/Z interact with *C. elegans* genes, it may occur through other mechanisms such as miRNA-like imperfect pairing (Zhao et al., 2017; Gu et al., 2017) or by targeting RNA-binding proteins as in Pseudomonas (Brencic et al., 2009).

Global transgenerational sRNA dynamics of the holobiont during infection

Gene expression of coding genes in *C. elegans* changes dramatically upon the encounter with new bacteria regardless of their pathogenic potential (MacNeil et al., 2013; Engelmann et al., 2011); (Radeke and Herman, 2020; Stuhr and Curran, 2020). Intergenerational transcriptomics (Data S1 and Gabaldón et al., 2020) show that sRNAs in bacteria and *C. elegans* also undergo drastic changes of expression upon their first encounter and are further modified in the second generation of exposure. Generation-specific changes in bacterial sRNAs (Figure 1C) appear relevant for the timing of PIDF and also suggest that bacteria and nematodes affect their gene expression bidirectionally throughout the generations. We asked how sRNA expression in the holobiont changes in a transgenerational scheme that spans for six generations (Figure 1A (Palominos et al., 2017), by dual-small RNA-seq (Westermann et al., 2012) of nematodes and their intestinal bacteria (Figure S5). Animals were fed on *P. aeruginosa* for two generations (F1-F2), the F3 was placed on non-pathogenic *E. coli* OP50 until the F4, and the F5 and F6 generations were re-exposed to *P. aeruginosa*.

Changes in sRNAs between naive and intestinal bacteria as well as throughout the generations in the holobiont were assessed using the DeSeq2 tool (Love et al., 2014). sRNA genes differentially expressed in intestinal *P. aeruginosa* and *E. coli* compared to their naive states are shown in Figures 3A and 3B, respectively. sRNA genes differentially expressed in *C. elegans* feeding on *P. aeruginosa* (F1 and F2), in *E. coli* OP50 after pathogen removal (F3 and F4) and after re-exposure to pathogens (F5 and F6), were obtained in comparison to animals continuously feeding on *E. coli* OP50 (Figure 3C). Our annotation was based on Transcriptional Peaks (TP, Gabaldón et al., 2020). To filter out the TPs potentially products of random degradation, we applied the moving median method (Liang et al., 2016); (Hardle and Steiger, 1995); https://www.R-project.org). The pertinence of using the TP method for bacteria and nematodes is explained in STAR Methods section.

After the first encounter with the *C. elegans* intestine, most gene expression changes in *P. aeruginosa* are repressive, a trend that is maintained over the generations (Figures 3D and 3E). In *C. elegans*, however, the largest changes occur in the F1 or upon the re-encounter with pathogens in the F5 (Figures 3D and 3F), suggesting the change from *E. coli* OP50 to Pseudomonas impacts dramatically gene expression.

The second exposure to *P. aeruginosa* in the F5, however, reveals specific changes that could be meaningful for the transgenerational memory. Bacterial ncRNAs differentially expressed in the F5 versus the F1 are shown in Table 2. 17 transcripts were differentially expressed between the two conditions, comprising ncRNAs, tRNAs, rRNAs, and novel TPs, suggesting a role of these RNAs in the transgenerational memory of previous exposures. In the subsequent generations (F2 and F6), global TP changes are smaller, suggesting a gradual adjustment between the two species over the course of interaction. A number of *C. elegans* TPs remained differentially expressed upon pathogen removal in the F3 (Figures 3D and 3F) either suggesting i) an intergenerational effect of pathogen exposure, ii) a soma to germline transmission of RNA of the F1 exposed to pathogens, or iii) an *in utero* effect on the F2 embryos of animals fed on pathogens. Analysis of individual TPs along generations reveals generation specific changes, as well as genes in expression maintained in successive generations (Table S2). This suggests that some genes respond to the pathogen encounter and others to the memory of such exposure in ancestors. Intestinal *E. coli* introduced in the

Table 2. Bacterial sRNAs genes are differentially expressed in the intestines of *C. elegans* F5 versus the F1 generations

Gene name	Biotype	Genomic context	Log2FC	padj
Upregulated in F5				
PA2581.1	tRNA	Match	2,2	6,E−03
rgsA	ncRNA	Nested	2,2	2,E−02
phrS	rRNA	Nested	2,1	2,E−02
PA0668.4	tRNA	Nested	4,6	3,E−02
PA5369.4	tRNA	Nested	3,4	8,E−03
PA4690.4	tRNA	Nested	3,1	2,E−02
PA0668.2	tRNA	Nested	2,8	2,E−02
PA4541.3	tRNA	Nested	2,3	9,E−02
PAO_16,841	Novel	Novel	5,9	8,E−03
PAO_8219	Novel	Novel	2,4	4,E−02
PAO_4048	Novel	Novel	2,3	8,E−03
Downregulated in F5				
PA4280.2	rRNA	Nested	−2,3	4,E−03
PA5369.2	rRNA	Nested	−2,3	4,E−03
PA4690.2	rRNA	Nested	−2,3	3,E−03
PA0668.4	rRNA	Nested	−2,3	3,E−03
crcZ	ncRNA	Nested	−2,5	2,E−03
mvaT	mRNA	Overlapped	−4,3	1,E−04

For novel genes, the gene name is our own ID.

F3 progeny of F2 animals fed with *P. aeruginosa* also represses most TPs (Figure 3G). Differential expression of intestinal *E. coli* in the F4 is very small indicating that it becomes transcriptionally similar to the *E. coli* of animals routinely exposed to this bacterium.

In intestinal *P. aeruginosa*, most repressed TPs are novel or nested in coding genes while most upregulated TPs are nested in previously annotated noncoding genes (Figure 3E). The most represented biotypes of noncoding RNAs were rRNAs and tRNAs but also include sRNAs and tmRNAs (Figures 3H and 3I). In *C. elegans*, noncoding genes were almost exclusively downregulated and included miRNAs, snoRNAs, rRNAs, piRNAs, and snRNAs (Figures 3J and 3K). Interestingly, TPs that matched previous annotation in bacteria are exclusively tRNAs and in nematodes tRNAs, snRNAs, and snoRNA (Table S3).

Both host-naive and intestinal bacteria and nematodes express a high number of previously unreported TPs (Figure S6), in coherence with previous reports of extensive pervasive transcription (Lybecker et al., 2014; Leitão et al., 2020). Many of these TPs are differentially regulated in many generations, suggesting they are bona fide transcripts although further studies are needed to confirm their functionality.

Histone modifications are required for PIDF and diapause transgenerational memory

The transgenerational memory of pathogens that induces dauer formation lasts for five generations in the absence of pathogens (Palominos et al., 2017). sRNAs and chromatin modifications are effectors of transgenerational inheritance (Rechavi et al., 2014; Houri-Ze'evi and Rechavi, 2016). We explored whether reported transgenerational mediators are needed for PIDF memory after pathogen withdrawal. We tested mutant animals of histone modifications known to participate in transgenerational inheritance such as the histone methyltransferases MET-2, SET-25, SET-32, SET-18, the chromatin-binding protein HPL-2 (Lev et al., 2017; Kalinava et al., 2017), and the argonaute HRDE-1 (Buckley et al., 2012).

In *C. elegans*, RNAi inheritance is known to depend on H3K9me3 (Lev et al., 2019). H3K9 is mono and dimethylated by MET-2 first and then trimethylated by SET-25 (Towbin et al., 2012). In the germline, SET-25 alone is enough for trimethylation of H3K9 (Bessler et al., 2010; Towbin et al., 2012). In the nuclei of germ

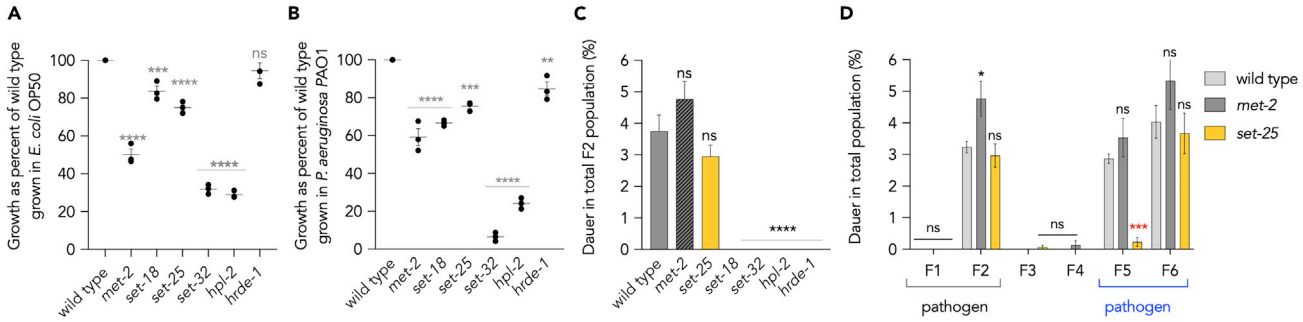

Figure 4. Specific histone methyltransferases required for PIDF initiation and memory maintenance in the holobiont

(A and B) Growth of HMT mutants on *E. coli* (A) and *P. aeruginosa* (B) as percent of the wild-type animals.

(C) Dauer formation of HMT mutants in the second generation.

(D) Transgenerational dauer formation of HMT mutants. Data are represented as mean ± SEM. p-value *<0.0332, **<0.0021, ***<0.0002, ****<0.0001.

cells, HRDE-1 directs the trimethylation of H3K9 at RNAi-targeted genomic loci and promotes RNAi inheritance. Candidates important for the execution of the dauer decision would fail to enter diapause in the F2 feeding on pathogens. On the other hand, effectors of the transgenerational memory of pathogens would form normal amounts of dauers in the F2 but not in the F5, after re-exposure to pathogenic bacteria. *set-32* and *hpl-2* mutants had impaired growth in non-pathogenic and pathogenic bacteria for two generations, so were unsuitable for PIDF testing (Figures 4A and 4B). *met-2*, *set-18*, *hrde-1*, and *set-25* mutations supported growth in both bacteria. We quantified the ability of *met-2*, *set-18*, *set-25*, and *hrde-1* to form dauers in the second generation when fed *P. aeruginosa* PAO1. While *met-2* and *set-25* animals formed normal amounts of dauers, *set-18* and *hrde-1* animals were unable to do so. The need for SET-18 suggests that di-methylation of H3K36 is important to trigger PIDF. Additionally, the requirement of HRDE-1 highlights that germline H3K9me3 and RNAi inheritance are key for PIDF (Figure 4C). To determine the role of *met-2* and *set-25* in transgenerational PIDF, mutants were exposed to pathogens in a transgenerational paradigm (Figure 1A). *met-2* animals performed wild-type transgenerational PIDF but *set-25* mutants failed to form dauers in the F5 (Figure 4D), suggesting a role for H3K9 in the transgenerational memory of pathogen exposure. Taken together, these results point out that diverse interspecies players, sRNAs and HMTs, contribute to first establishing PIDF and posteriorly eliciting the memory of the past bacterial encounter in subsequent generations.

DISCUSSION

Understanding microbiome-induced inheritance is of broad relevance for uncovering the transgenerational origins of health and disease. Here, we demonstrate that sRNAs of intestinal pathogens and host histone modifications are required for a transgenerational adaptive behavior in *C. elegans*. Specifically, bacterial *RsmZ* and *RsmY*—both quorum sensing activators—are upregulated sequentially during the colonization of the nematode intestine. *RsmZ* is expressed early and steadily during the interaction while *RsmY* is overexpressed in the second generation of nematodes undergoing pathogenesis. Importantly, RsmY is needed for diapause formation under pathogenesis and its role is key in the second generation. RsmZ, however, is not essential but required for the wild-type behavioral response. Furthermore, RsmY expressed heterologously promotes PIDF only if RsmZ is present. At the chromatin level, the histone methyltransferase SET-18 and the argonaute HRDE-1 are necessary for the initiation of the behavior. The H3K9 trimethyltransferase SET-25 is necessary for the elicitation of the memory of pathogenic encounters. In sum, this work unveils interspecies sRNA dynamics through generations of interaction and histone modifications relevant for the establishment of an adaptive behavioral strategy between interacting species.

Specific bacterial sRNAs are able to drastically affect host behavior

In our study, we found a new role for the bacterial sRNA RsmY in interspecies communication. RsmY is a conserved noncoding RNA in *P. aeruginosa* with a role in communication between bacteria, as quorum sensing (QS) and biofilm regulator (Kay et al., 2006). In *P. aeruginosa*, RsmY and RsmZ are activated by the GacS/GacA system. Both bind and antagonize the RNA-binding protein RsmA, which negatively controls the expression of quorum sensing and several extracellular products (Pessi et al., 2001; Kay

et al., 2006). Our results show that RsmY and RsmZ have different roles in PIDF. We propose that these two RNAs act sequentially at successive colonization times. RsmZ is induced early in the interaction with the host, and RsmY is expressed in the second generation as a result of sustained interaction. Other Pseudomonas strains capable of inducing behavioral aversion in *C. elegans* also express RsmZ under stress in naive conditions (Kaletsky et al., 2020). We reasoned that these two RNAs could act restricted to the intestine or be internalized to promote an effect in *C. elegans* tissues far from the intestinal lumen. In the first scenario, these sRNAs would impact the lifestyle of intestinal bacteria by affecting the biofilm formation dynamic and therefore impacting intestinal physiology [e.i intestinal distention (Hong et al., 2021)]. In the second scenario, bacterial sRNAs could be internalized through specific dsRNA transporters in the intestine or through outer membrane vesicles (OMVs). By either mechanism, sRNAs could reach other organs where direct molecular interactions between bacteria and worm transcripts would occur, using the RNAi machinery of the host. In this scenario, transcriptional changes could impact the germline of exposed parents and affect their progeny even when they are not in direct contact with bacteria (Rechavi and Lev, 2017; Nono et al., 2020; Hong et al., 2021). These findings broaden our understanding of how sRNAs of colonizing bacteria such as the microbiota regulate host behavior.

Small RNA dynamics in the holobiont across generations

While host bacterial-RNA targets have been found to promote infection (Koeppen et al., 2016; Zhao et al., 2017), there is scarce evidence about how exogenous sRNAs from the microbiota affect physiology and its inheritance. This work reveals that bacterial sRNAs are essential for the host response to the pathogen.

sRNA expression fluctuated in the holobiont throughout the generations of interactions between nematodes and bacteria. This suggests that a bidirectional regulation of RNA expression is established and depends on the nature of bacteria and the time of residence in the nematode intestine. For example, specific bacterial sRNAs were overexpressed in the second generation of animals suggesting their expression arises from the active co-regulation with the host. We focused on these specific RNAs and their role in triggering PIDF, which is established in the second generation. Additionally, bacterial sRNA expression responds dynamically to changes in gene expression in the host throughout the different generations. In fact, bacteria colonizing animals whose progenitors have been exposed for more than two generation to the pathogen (F5), also express different sRNA signatures than in the first time of exposure. This suggests that bacteria can respond to the host memory of previous experiences with, according to a transactional paradigm, in which the transcriptional state *post-interaction* alters the gene expression of upcoming interactions.

One advantage of performing whole organism dual-RNAseq is to understand systemic responses during the pathogen-host interplay. Using this strategy, Zhang et al. (Zhang et al., 2020) found that the two-component system BarA/UvrY from *Vibrio parahaemolyticus* is important for virulence. We aimed to understand long-term behavioral changes rather than virulence, so the challenge was to discriminate the transcriptional changes over time, which are not directly related only to infection. This is the first work that evaluates the whole organism interplay with bacteria over different generations.

Histone modifications are necessary for initiation of behavior and transgenerational memory

The evidence of microbiome-induced phenotypic changes in hosts is increasing, but there is scarce evidence of the long-term epigenetic changes triggered by bacteria. Previously, we showed that the interaction with pathogens induces a memory that is inherited transgenerationally (Palominos et al., 2017). This requires that epigenetic information is transmitted by the germline in the absence of direct environmental exposure (Skinner, 2011; Jablonka and Raz, 2009). In our paradigm, the pathogen exposure was maintained for two generations, and then withdrawn for two more generations. The first generation after withdrawal (F3) grew in the uterus of an infected mother, so we could see transcriptomic changes that reflect embryonic exposure. In the following generation, the F4, there is no possibility of direct exposure. We did not find either *C. elegans* or bacterial sRNAs that persist in the F4, suggesting other transgenerational effectors may play a role in this transmission. In our paradigm, the transgenerational memory is observed after re-exposure to pathogens, where animals form dauers immediately in contrast with the two generations needed the first-time animals are fed pathogens. We found that the H3K9 trimethyl transferase SET-25 is necessary for either storage or retrieval of the inherited memory, even though it is dispensable for

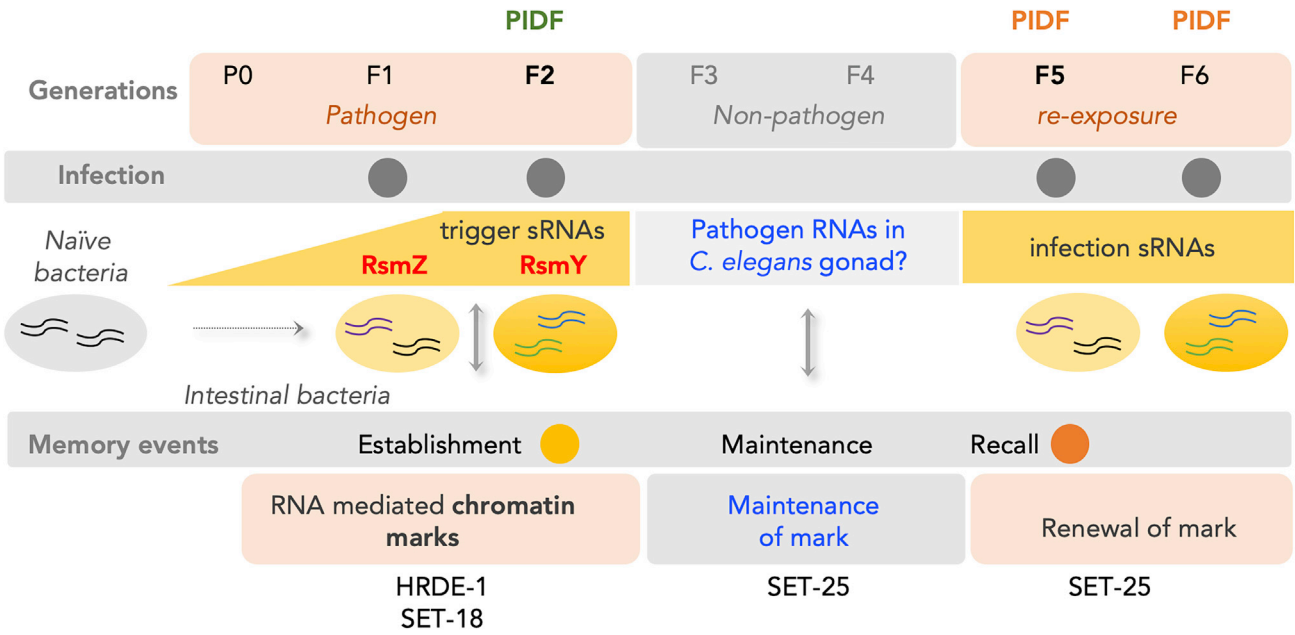

Figure 5. Model of integrated multistep rationale of PIDF pathogenic memory

Specific changes in *C. elegans* and its intestinal bacteria occurring in each generation giving rise to PIDF and memory formation.

intergenerational PIDF. SET-25 was previously reported to be necessary for the initiation but dispensable for transgenerational maintenance of RNAi silencing in other paradigms (Woodhouse and Ashe, 2019). On the other hand, H3K9me3 is not required for nuclear RNAi-induced transcriptional silencing (Kalinava et al., 2017; Mao et al., 2015). In our paradigm, however, HRDE-1 is required for PIDF, confirming that dauer induced by pathogenesis is triggered by exogenous dsRNA and needs germline RNAi (Palominos et al., 2017). In sum, these results suggest that the participation of H3K9me3 in transgenerational inheritance may vary depending on the genes and the phenomena studied.

Integrated multistep rationale of PIDF pathogenic memory

PIDF is a multistep strategy that first includes the establishment of the defensive strategy in the second generation, and later, its maintenance from the F3 onward (Figure 5). Many players are involved in these two steps: sRNAs from bacteria and host, and multiple tissue-specific transcriptomic and epigenetic changes. Our results previous and current suggest that animals need to be actively infected by the pathogen, a process that includes the intestinal expression of RsmZ first and then RsmY in the second generation. The establishment of the pathogenic state triggers epigenetic changes that require HMTs such as HRDE-1 and SET-18. During the removal of the pathogen, these epigenetic marks may remain due to the action of SET-25 and allow the recall of memory immediately upon re-exposure.

Limitations of the study

We demonstrate the effect of bacterial RNA *rsmY* in inducing dauer formation under pathogenesis. Presently, however, we have not yet determined whether this effect is directly or indirectly affecting the host physiology. A step forward to answer this would be the evaluation of *rsmY* presence in *P. aeruginosa* OMVs, or using fluorescent probes to localize this particular RNA during Pseudomonas colonization of *C. elegans*. This specific question is limited by the availability of labeling techniques for transcripts of small size. Additionally, we excluded from our analysis some gene expression regulatory layers such as bacterial mRNA and we focused only on small RNAs. This was a hypothesis-driven decision, because of the evidence of RNAi participation in the phenomenon. Likewise, we selected the histone modifiers of our study biasedly, based on literature or theoretical mechanisms. So, this was not a wide screening of possible mechanisms. Thus, we cannot exclude other relevant players participating in PIDF.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104627.

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AUTHOR CONTRIBUTIONS

Conceptualization ML and AC, Methodology ML, BP, and AC, Investigation ML, BP, BA, MC, and AC, Writing-Original Draft ML and AC, Writing-Review and Editing ML and AC, Funding Acquisition AC.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli OP50	Caenorhabditis Genetics Center	DA837
RNAi-competent *E. coli* rnc14::DTn10 and lacZgA::T7pol camFRT	Laboratory of Dr. Meng Wang	N/A
Pseudomonas aeruginosa PAO1 *wild type*	ATCC	ATCC15692
Pseudomonas aeruginosa PAO 6354 (*ΔrsmZ*),	Heurlier et al., 2004 (Laboratory of Stephan Heeb)	PAO 6354
Pseudomonas aeruginosa PAO 6420 (*ΔrsmY*)	Kay et al., 2006 (Laboratory of Dr. Stephan Heeb)	PAO 6420
Pseudomonas aeruginosa PAO 6421 (*ΔrsmY rsmZ*)	Kay et al., 2006 (Laboratory of Dr. Stephan Heeb)	PAO 6421
Critical commercial assays		
Ribogreen Assay Kit	Life Technologies	R11490
TRI-reagent	MRC	SKU: TR118
RNA 6000 Pico Kit	Agilent Technologies	N. 50671513
TRizol reagent	Invitrogen	N. 15596026
ImProm-II Reverse Transcriptase	Promega	A3800
KAPA HiFi HotStart ReadyMix 2X PCR Kit	Kapa Biosystems	N/A
UltraClean® Microbial DNA Isolation Kit	Mo Bio Laboratories Inc.	N/A
DNase I	Invitrogen	N. 18068015
Deposited data		
Raw data (intergenerational fastq files)	This study	SRA: PRJNA659467
Raw data (transgenerational fastq files)	This study	SRA: PRJNA708299
Pipeline- Scripts	This study	https://bitbucket.org/mlegue/workspace/projects/TSI
Experimental models: Organisms/strains		
C. elegans: Strain N2	Caenorhabditis Genetics Center	N2
C. elegans: Strain MT17463 [*set-25(n5021)*]	Caenorhabditis Genetics Center	MT17463
C. elegans: VC967 [*set 32(ok1457)*]	Caenorhabditis Genetics Center	VC967
C. elegans: VC767 [*set-18(gk334)*]	Caenorhabditis Genetics Center	VC767
C. elegans: MT13293 [*met-2(n4256)*]	Caenorhabditis Genetics Center	MT13293
C. elegans: RB1090 [*hpl-2(ok1061)*]	Caenorhabditis Genetics Center	RB1090
C. elegans: TJ356(*zIs356* [*daf-16p::daf-16a/b*::GFP + *rol-6(su1006)*	Caenorhabditis Genetics Center	TJ356
Oligonucleotides		
Primers for RsmY: 5′ AGGACATTGCGCAGGAAG 3′ and y 5′ GGGGTTTTGCAGACCTCTATC	This study	N/A
Primers for RsmZ: 5′ CGTACAGGGAACACGCAAC 3′, and 5′ TATTACCCCGCCCACTCTTC 3′	This study	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Cutadapt	Martin, 2011	N/A
BEDTools	Quinlan and Hall, 2010	N/A
FeatureCounts	Liao et al., 2014	N/A
Bioconductor Rsubread package	Anders et al., 2015	N/A
Blast +	Camacho et al., 2009	N/A
SAMTools version 0.1.19		N/A
DeSeq2	Love et al., 2014	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts Andrea Calixto (andrea.calixto@uv.cl) and Marcela Legüe (marcelegue@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Fastq files from intergenerational Dual-RNA-seq and from intergenerational Dual-RNA-seq data have been deposited at NCBI Sequence Read Archive (SRA) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- All original code has been deposited at Bitbucket and will be shared by the lead contact upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans

Wild type (N2) hermaphrodite nematodes and mutant strains MT17463 [*set-25(n5021)*], VC967 [*set-32(ok1457)*], VC767 [*set-18(gk334)*], MT13293 [*met-2(n4256)*], RB1090 [*hpl-2(ok1061)*], YY538 [*hrde-1(tm1200)*] and TJ356 (zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]) were grown at 20°C (Brenner, 1974). All nematode strains were maintained on *E. coli* OP50 strain prior to feeding with other bacteria. We used synchronized populations of nematodes to start the experiments and for the scoring. Details are provided below for each case.

Bacteria

The following bacterial strains were used as *C. elegans* food: *E. coli* OP50, RNAi-competent *E. coli* rnc14::DTn10 and lacZgA::T7pol camFRT, *P. aeruginosa* PAO1, PAO6354 *(ΔrsmZ)*, PAO6420 *(ΔrsmY)* and PAO6421 *(ΔrsmY rsmZ)*. Bacteria were grown overnight on Luria-Bertani (LB) plates at 37°C from glycerol stocks. The next morning a scoop of the bacterial lawn was inoculated in LB broth with antibiotics (streptomycin for *E. coli* OP50 and grown for 6 h on agitation at 450 g at 37°C (OD_{600} 1.5–2.0). A volume of 3 mL of this bacterial culture was seeded onto 90 mm NGM plates and allowed to dry overnight before nematodes are placed on them.

METHOD DETAILS

Dual sRNA-seq sample preparation

LB cultured (naïve) bacteria

Bacterial inoculums of *E. coli* OP50, *P. aeruginosa* PAO1, PAO6354 *(ΔrsmZ)*, PAO6420 *(ΔrsmY)* and PAO6421 *(ΔrsmY rsmZ)* were taken from glycerol stocks kept at −80°C and grown overnight on

LB agar plates at 37°C (with streptomycin 100 μg/mL or without antibiotic, depending on the bacterial strain). Next morning, a scoop of the bacterial lawn was inoculated in 15 mL of liquid LB with the same antibiotics, at 37°C in agitation for 4 h or until they reached an OD_{600} between 1.5 and -2.0. A volume of 1.5 mL of the sample was pelletized and incubated with 200 μL of pre-heated TRIzol™ Max™ Bacterial Enhancement Reagent (Thermo-Fisher) at 95°C for 4 min for posterior RNA extraction.

Bacteria in C. elegans intestine in the intergenerational setting

Wild-type *C. elegans* were cultured on 60-mm-diameter Petri dishes with NGM medium seeded with 50 μL of *E. coli* OP50 and maintained at 20°C. After 3 days, mixed stage animals were treated with bleaching solution and embryos were deposited in new plates. Forty-eight hours later, most individuals were in the L4 stage. Five L4 worms were transferred to 90 mm plates previously seeded with 3 mL of *E. coli* OP50 or *P. aeruginosa* PAO1. In all cases the bacterial lawn covered the plate. Worms were allowed to grow at 20°C for 24 h. After that time, all animals on the plate were subjected to hypochlorite treatment. F1 embryos were collected in 1 mL of M9 and centrifuged at 394 g. The embryos obtained were placed on a new 90 mm plate with 3 mL of bacteria. After 24 h, animals were collected with M9 for total RNA extraction. Worms on the other three plates were allowed to grow for another 48 h until the F1 was gravid. The F2 progeny was collected equally as the F1 and placed on plates seeded with bacteria cultured for the same time and under the same conditions as the F1. Animals were collected for RNA extraction 24 h later. Each condition was performed in triplicates obtaining a total of 12 samples (F1 and F2 in *E. coli* OP50 and *P. aeruginosa* PAO1).

C. elegans and its intestinal bacteria in the transgenerational setting

Wild type *C. elegans* were cultured on standard 60 mm diameter Petri dishes with NGM media, seeded with 0.5 mL of *E. coli* OP50 and maintained at 20°C. After three days, a decontaminant treatment with NaOH and sodium hypochlorite was applied (Stiernagle, 2006). As a result, only clean embryos were deposited in new dishes. After 48 h, most individuals were in the L4 stage. 20 to 25 L4 worms were transferred to 90 mm NGM plates previously seeded with 3 mL of *E. coli* OP50 or *P. aeruginosa* PAO1. In all cases the bacterial lawn covers the plate. Worms were allowed to grow at 20°C for 48 h. The resulting population was collected with an M9 buffer and treated with sodium hypochlorite to collect only embryos (F1) that were allowed to grow for 24 h under the same conditions as their progenitors (P0). Half of the F1 larvae were washed with M9 ten times to avoid external bacteria from the worm body, and frozen at −80°C for posterior RNA extraction. The other half was allowed to grow under control or pathogenic conditions for 2 more days (F1 adults). F2 embryos were obtained and grown exactly as the F1. L1-L2 larvae were harvested with an M9 buffer, washed with M9 ten times to avoid external bacteria from the worm body, pelletized and frozen at −80°C for RNA extraction. Each condition was performed in triplicates. Frozen samples of L1-L2 larvae from F1 and F2 are pre-processed for mechanical lysis by vortexing the sample with 0.5 mm steel beads for five minutes. RNA purification from pelleted worms is performed using phenol-chloroform extraction (TRI reagent®, Sigma-Aldrich) with the same protocol described above. Our experiment consisted of 18 samples: 6 RNA samples from worms grown on *E. coli* OP50 or *P. aeruginosa* PAO1. For each bacterium 3 samples were from the F1 and 3 from the F2 generations. Quality tests were performed by Nanodrop, Denaturing gel electrophoresis and finally using DNA/RNA 1000 chip kit in a Bioanalyzer 2,100 (Agilent Technologies) and quantified by Quant-iT™ RiboGreen® Assay kit (Life Technologies).

Small RNA extraction of *naïve* bacteria and bacteria in *C. elegans* intestine

1 mL of TRI-reagent (MRC) was added to the pellet sample and one steel bead of 0.5 mm was added to the tube. Maximal vortex was applied for 5 min; the sample was centrifuged and changed to a new tube. Phase separation was performed adding 200 μL of chloroform and vortexing. After centrifugation at 12,000 g for 15 min at 4°C, 400 μL of aqueous RNA phase was precipitated at room temperature overnight, followed by centrifugation for 15 min at 4°C. The supernatant was discarded, and the pellet was washed with EtOH 75%, air-dried for 10 min and resuspended in 20 μL of DNAse-free water. DNase I treatment was performed adding 2 μL of buffer, 2U of DNAse I, and incubated at 37°C for 10 min. Inactivation of DNAse was done with the addition of 2 μL of 50 mM EDTA. Each procedure was performed in triplicates. Size selection was performed to keep fragments shorter than 200 nt. Control of sizing, quantification and quality was made with Agilent 2,100 Bioanalyzer, using RNA 6000 Pico kit.

Small RNA sequencing

cDNA library preparation was made with TruSeq Small RNA Library Preparation Kits for naive and intergenerational samples and with Diagenode CATS Small RNA sequencing kit for Illumina for transgenerational samples, according to manufacturer's protocol. All RNA-seq experiments generated Illumina reads in FASTQ format (Key resources table).

RNA extraction in a transgenerational paradigm

60 L4 worms (P0) were placed on plates seeded with *P. aeruginosa* PAO1. After 24 h the plates were carefully washed with M9 to eliminate larvae and adult worms. The laid eggs (F1) were allowed to grow for 24 h in *P. aeruginosa* PAO1. F1 L2s were collected with M9 in an Eppendorf tube and washed 5 times with 1 mL of M9 and centrifugation at 2000 rpm. The L2 F1 worms were subjected to total RNA extraction with TRiZol reagent (Invitrogen).

To obtain F2 worms, 20 L4 (P0) worms were seeded on a plate with *P. aeruginosa* PAO1 and allowed to grow for two generations for 7 days until the first F2 embryos were laid. F1 gravid hermaphrodites were treated with hypochlorite and F2 embryos were placed on a plate with fresh PAO1 and allowed to hatch and grow for 24 h. L2 animals were collected for RNA extraction. A portion of the F2 embryos was placed in a *P. aeruginosa* PAO1 plate until worms reached adulthood. Animals were treated with hypochlorite and their F3 was placed on a plate with *E. coli* OP50. After 24 h, worms were collected for RNA extraction. A part of F3 embryos were maintained in *E. coli* OP50 until adulthood. F4 embryos were obtained by hypochlorite treatment and allowed to grow for 24 h when L2 were collected for RNA extraction. A part of F4 embryos were maintained in *E. coli* OP50 until adulthood. Gravid hermaphrodites were then treated with hypochlorite solution and their eggs placed on a fresh plate with *P. aeruginosa* PAO1 for 24 h. L2 worms were washed, and their total RNA extracted. A part of F5 embryos was placed in a plate with *P. aeruginosa* PAO1 until obtaining embryos of the sixth generation. F5 hermaphrodites were treated with hypochlorite and the F6 embryos were placed on fresh *P. aeruginosa* PAO1 plate for 24 h. 24 h later, L2 animals were collected for extraction of total RNA.

Bioinformatic analysis

Quality control

Quality visualization was made with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Data pre-processing

Trimming was performed with Cutadapt (Martin, 2011), applying Diagenode recommended executable script CATS_trimming_r1.sh for CATS Library Preparation Kits available at https://www.diagenode.com/en/documents/diagenode-trimming-tools-for-cats-rnaseq.

Mapping and annotation

For each sample, reads were mapped to *E. coli* OP50 genome assembly ASM435501v1, *P. aeruginosa* PAO1 ASM676v1 or *C. elegans* WS267. Alignment was performed with Bowtie2 version 2.2.6 (Langmead and Salzberg, 2012) with one allowed mismatch and seed set to 17 bp. As a result, a bam file was produced for each sample.

TPs detection

We selected those genomic areas covered by mapped reads to define customized features, named transcriptional peaks (TPs) defined as a genomic area that shows a peak of expression. TPs were defined as features between 17 and 150 nucleotides with an average coverage of 10 or more reads by nucleotides. This was done by merging bam files from each sample using SAMTools version 0.1.19. The features obtained for both strands were gathered and sorted to create a custom GFF file for further analysis. Next, we counted the reads against those custom GFF files. For comparison with databases, we intersected TPs with reported annotations and classified them according to their genomic context (Gabaldón et al., 2020).

Comparing annotated genes with TPs

We categorize our TPs as known (matching 85% or more of an annotated feature), novel (in intergenic regions), and partially novel (nested or overlapped to annotated features, with less and more than 15% of

unmatched nucleotides respectively). All of them are also categorized as sense or antisense to a known reference feature. To accomplish that, we compared our TPs against the Ensembl annotations of *C. elegans* PRJNA13758, WS267 by using the intersect function of BEDTools (Quinlan and Hall, 2010), adapted from Gabaldón et al. (2020). We performed the same process for *E. coli* OP50 ASM435501v1 and *P. aeruginosa* PAO1 ASM676v1 (Quinlan and Hall, 2010). We also created a TPs fasta file with the feature sequences by using getfasta BEDtools command.

Differential expression analysis

For each sample read count was performed with featureCounts (Liao et al., 2014) from bioconductor Rsubread package with default parameters (Anders et al., 2015). Then, the count matrix was used to perform differential expression analysis in R version 3.3.2 (Anders and Huber, 2010; Robinson et al., 2010) between the naïve and intestinal bacteria of each generation using DeSeq2 (Love et al., 2014). We use p adjusted (padj) values to discriminate differentially expressed genes with a cut-off value of 0.01.

For analysis in which differential expression is not involved, we generate normalized expression by applying the TMM method (Trimmed Mean of M, Robinson and Oshlack, 2010).

RsmY crosskingdom matching sequences

We searched for similar sequences between *rsmY* and a database generated with the *C. elegans* genome (WS267) using Blast + (Camacho et al., 2009).

Pertinence of the TP method. One of the goals of this work was to identify small RNA transcripts with a role in the interaction between microbe and nematode. Because transcription is context dependent, there is no previous evidence of the changes that will emerge from this interaction (We do not know how many fragments that are not annotated have relevance in interspecies communications). This warrants an unbiased analysis of transcriptional changes. To date there are no studies that perform small Dual RNAseq *in vivo*. Therefore, the possibility of finding non-annotated transcripts is higher compared to known conditions previously studied. This method tries to separate transcriptional noise (mix of transcription, aleatory cleavage) from true transcripts. This was based on taking varied replicas under which (10nt) the rest is eliminated. Only statistically significant TPs remain. We performed the TP-based analysis in the three species studied for the following reasons: a) to normalize we need to compare similar methods, and b) it allowed the discrimination of similar fragments (not transcripts) between species (Legüe et al., 2021).

Quantification of dauer formation and population size

In the second generation of pathogen exposure

Entire worm populations on each plate were collected in 1 mL of M9. The initial stock was diluted at 1:10 in M9. The total population including dauer larvae was treated with 1% SDS for 20 min (Cassada and Russell, 1975). A volume of 10 μL of each dilution was used to count the total population (number of corpses) and dauer larvae (number of living dauers) respectively under a Nikon SMZ745 stereomicroscope. Each technical replica was scored 3 times and the number of dauers was plotted as percent of the total population of animals.

Transgenerational dauer formation

Quantification of dauers was done as in Palominos et al. (2017); and Chávez and Calixto (2019) with minor variations. Control animals were maintained on *E. coli* OP50 for six generations, while test animals were exposed to *P. aeruginosa* PAO1 for two generations, passed as F3 embryos to *E. coli* OP50 until the F4, and reintroduced to *P. aeruginosa* PAO1 in the F5 and F6 generations. In detail, we exposed 15 L4 animals (P0) to *P. aeruginosa* PAO1 or *E. coli* OP50 on 90 mm plates. 24 h later, gravid adults were isolated and treated with sodium hypochlorite (Stiernagle, 2006). As a result, nearly 6,000 clean embryos were deposited in new Petri dishes. This process was made every three days in animals fed on *E. coli* OP50 and every four days on *P. aeruginosa* PAO1. F2 adults were treated with sodium hypochlorite, and the embryos were passed to new plates of *E. coli* OP50. The same treatment was done with the F3 and the F4 generations. F5 embryos from bleached F4 adults were passed to pathogens, and their F6 progeny as well, using the same treatments. Embryos on *P. aeruginosa* PAO1 were allowed to grow for 48–72 h before treating the entire plate with 1% SDS for the quantification of dauers. All experiments were done in three biological replicates of three technical replicates each.

Importantly, six plates were used for each condition; three were used for dauer counts and three for hypochlorite treatment of adults.

Pathogen avoidance

60 mm NGM plates were seeded with 400 μL of bacteria inoculum incubated at 37°C in agitation for 4 h. Next day, 30 N2 worms were transferred by picking and placed in the center of the lawn. The number of worms inside and outside the lawn was quantified at 10 and 30 min, 1 h, 2 h and 24 h.

Quantification of intestinal colony forming units

Thirty L4 animals were picked into an Eppendorf tube containing M9 buffer with 25 mM levamisole hydrochloride (Sigma-Aldrich) to cause paralysis and stop pharyngeal pumping (Kawli and Tan, 2008). The animals were then washed three times with M9 containing 1 mg/mL gentamicin (Sigma-Aldrich) and 1 mg/mL ampicillin (Sigma-Aldrich). After the third wash, the animals were incubated once more with the antibiotic mixture for 1 h. To eliminate the antibiotic, the animals were washed three more times with M9 containing 25 mM levamisole. Each worm pellet was lysed with an individual pestle, and the resulting lysate was serially diluted 1:10 seven times in M9. Amounts of 200 of each dilution were individually plated on LB with streptomycin (to select *E. coli* OP50), and without antibiotics for *P. aeruginosa* PAO1. The plated dilutions were incubated overnight at 37°C. The amount of CFUs was calculated using the following formula: CFU per worm/[(colonies per plate/dilution factor)/plated volume]/number of worms.

Reverse transcription of bacterial RNA

Two μg of total RNA were treated with DNase I (Invitrogen) according to the manufacturer's instructions. ImProm-II Reverse Transcriptase (Promega) was used to synthesize cDNA from total RNA. Briefly, 1 μg of total RNA with 0.5 μg of random primers (Promega) and distilled water up to 10 μL of final volume were incubated at 70°C for 5 min and then kept on ice for 5 min. The reverse transcription mix was prepared with 4 μL ImProm-II 5X Reaction Buffer, 2.5 mM $MgCl_2$, 0.75 mM dNTP mix and 1 μL of Super-Script IV Reverse Transcriptase (Thermo Fisher Scientific) for each sample and mixed with 10 μL of the previous reaction. Synthesis was performed in an Aeris Thermal Cycler using the following parameters: annealing at 25°C for 5 min, extension at 42°C for 60 min, inactivation of the reverse transcriptase at 70°C for 15 min.

Confirmation of mutations in single and double mutants of *P. aeruginosa* PAO1 by PCR

To confirm RsmY and RsmZ expression in the mutant strains PAO6354 (Δ*rsmZ*), PAO6420 (Δ*rsmY*) and PAO6421 (*ΔrsmY rsmZ*), we used the following primers: For amplification of rsmY 5′-AGGACATTGCGCAGGAAG-3′ and 5′-GGGGTTTTGCAGACCTCTATC-3′; for rsmZ 5′-CGTACAGGGAACACGCAAC-3′, and 5′-TATTACCCCGCCCACTCTTC-3′. These primers were designed based on conserved regions of genes in *P. aeruginosa* PAO1 (GCF_000006765.1, *Pseudomonas* Genome Database (Winsor et al., 2016). PCR reaction mixtures consisted of 10 μL of KAPA HiFi HotStart ReadyMix 2X PCR Kit (Kapa Biosystems), 0.3 μM forward primer, 0.3 μM reverse primer, 100 ng of bacterial cDNA and water up to 20 μL of final volume. Amplification was performed in an Aeris thermal cycler using the following parameters: initial denaturation at 95°C for 3 min, 98°C for 20 s for each subsequent cycle; annealing 58°C for 15 s and elongation at 72°C for 15 s for 30 cycles; and a final elongation at 72°C for 1 min.

Amplicons from the PCR amplifications were purified using a QIAquick PCR Purification Kit (Qiagen, Cat No.28104). Sequencing of the PCR product was performed by Capillary Electrophoresis Sequencing (CES) for difficult samples (Macrogen Korea) using the same primers described before.

RsmY and RsmZ expression quantification in bacteria by Real Time PCR

RsmY and RsmZ expression quantification was done by RT-PCR using Mir-X miRNA qRT-PCR TB Green Kit (Takara Bio USA, Inc). For each reaction, 50 ng of bacterial cDNA, 12.5 μL of TB Green Advantage qPCR 2X, 0.5 μL ROX (50X) LSR, 0.2 μM primers described before (For amplification of rsmY 5′-AGGACATTGCGCAGGAAG-3′ and 5′-GGGGTTTTGCAGACCTCTATC-3′; for rsmZ 5′-CGTACAGGGAACACGCAAC-3′, and 5′-TATTACCCCGCCCACTCTTC-3′.For amplification of rsmY 5′-AGGACATTGCGCAGGAAG-3′ and 5′-GGGGTTTTGCAGACCTCTATC-3′; for rsmZ 5′-CGTACAGGGAACACGCAAC-3′, and 5′-TATTACCCCGCCCACTCTTC-3′), and water up to 25 μL final volume was used. The StepOnePlus Real-Time PCR System was used with the following conditions: Initial denaturation at 95°C for 3 min, 30 cycles of 98°C for 20 s, 58°C for 15 s and 72°C for 15 s, followed by a final extension at 72°C for 1 min.

Bacterial DNA fragment amplification

DNA extraction of *P. aeruginosa* PAO1 was performed using Ultra-Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc.) according to the manufacturer's instructions. Amplifications of *rsmY* and *rsmZ* genes were performed by PCR (same PCR conditions as previously mentioned in confirmation of mutant strains section) using genomic DNA as template. PCR products were run on 2% agarose gel electrophoresis. Thereafter, fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Cat No.28104) and quantified in Infinite 200 PRO NanoQuant. Finally, DNA fragments were stored at a concentration of 16.66 fmol/μL at −20°C until used.

Construction of L4440-rsmY and L4440-rsmZ

A 500 ng mass of L4440 vector (Fire et al., 1998) was digested using EcoRV (NEB Cat. No. R0195S) and dephosphorylated using Antarctic Phosphatase (NEB Cat. No. M0289S) according to the manufacturer's instructions to generate a linearized blunt-ended fragment for cloning. RsmY and RsmZ were amplified by PCR using oligonucleotides containing flanking SapI restriction enzyme sites. For amplification of rsmY, 5′AAGCTCTTCAGGTGTCAGGACATTGCGCAGG-3′ and 5′TTGCTCTTCTGTAAAAACCCCGCCTTTTGG-3′ were used, and for amplification of rsmZ 5′AAGCTCTTCAGGTCGTACAGGGAACACGCAAC-3′ and 5′TTGCTCTTCTGTAAAAAAGGGGCGGGGTATTAC-3′ were used. PCR amplicons were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega Cat. No. A9281). Next, a mass of 200 ng of the amplicons was digested using SapI (NEB), followed by a single elongation step PCR by adding an equal volume of previously prepared Phusion polymerase PCR reaction mix at 2X final concentration (Phusion High-Fidelity DNA Polymerase, ThermoFisher Cat. No F-530S) without oligonucleotides. This provided 5′ phosphate-containing blunt fragments for cloning. 5′ phosphate-containing rsmY and rsmZ amplicons were then ligated to the linearized dephosphorylated L4440 vector using a 2-h room temperature incubation with T4 DNA ligase (NEB Cat. No. M0202S) according to the manufacturer's instructions. The samples were then transformed into TOP10 chemically competent cells and plated onto LB agar plates containing appropriate antibiotics. Resulting colonies were screened by colony PCR, purified by column-purification (Qiagen) and verified by Sanger sequencing (Macrogen Inc., Korea).

Bacterial transformation

A volume of 100 μL of chemically competent TOP10 cells (Thermo Fisher Scientific), were transformed with 2 μL of L4440-rsmY or L4440-rsmZ plasmid from the previous step and kept incubating on ice for 30 min. Thereafter heat-shock was performed for 45 s at 42°C and immediately placed on ice for 5 min. 900 μL of LB medium were added to the previous mix and the tube was incubated at 37°C for 2 h at 200 rpm. Then, 50 μL of each reaction were plated on LB agar plates with 100 μg/mL ampicillin, 40 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal, Thermo Scientific) and 1 mM isopropyl beta-D-thiogalactopyranoside (IPTG, US Biological). Plates were incubated at 37°C for 24 h. For bacterial colony screening, two white positive colonies were selected for each plate and grown in 5 mL of LB medium with 100 μg/mL of ampicillin and incubated at 37°C for 24 h.

Plasmids from TOP10 cells positive colonies were extracted using QIAprep Spin Miniprep kit and sequenced at Macrogen Korea. Additionally, constructs were also digested with BsaI enzyme (New England Biolabs), using 1 μL Cutsmart buffer 10x, 0.2 μL BsaI (10U/μL), 100 ng of plasmid and water up to 10 μL final volume. Reactions were incubated at 37°C for 1 h. Subsequently, the reaction was run on 1.5% agarose gel electrophoresis to verify the presence of fragments.

RNAi-competent *E. coli* rnc14:DTn10 and lacZgA:T7pol camFRT and PAO6420 (Δ*rsmY*) were transformed using the same procedure as TOP10. To check RsmY expression from the L4440-RsmY construct, the RNA extraction was performed with TRi-Zol reagent (Invitrogen). Afterwards reverse transcription of bacterial RNA was done and finally PCR using confirmation of *rsmY* amplification as described above. To check rsmZ expression from RNAi-competent *E. coli* rnc14:DTn10 and lacZgA:T7pol camFRT, PCR of selected colonies were performed. Single colonies were selected and placed in 20 μL of sterile water. Bacteria was lysed to release DNA at 95°C for 10 min. Bacterial lysate was used as a template for PCR reaction with specific primers to RsmZ.

QUANTIFICATION AND STATISTICAL ANALYSIS

Experimental replicas and statistical evaluation

All experiments were done at least 3 times (three biological replicas, started in different days and from different starting plates). Each biological replica contained a triplicate (three technical replicas).

Data in graphs are represented as mean ± SEM. Figure with asterisks represent p-values as follows: *<0.0332, **<0.0021, ***<0.0002, ****<0.0001. Statistical evaluation was done by a one or two-way ANOVA with post-hoc analyses. Results of all tests are detailed in Source Data S1.

Criteria for data exclusion

We excluded experimental replicas when there was contamination with unwanted bacteria or fungi on the nematode plates, or when bacteria had been almost or completely consumed.