Intergenerational pathogen-induced diapause in *C. elegans* is modulated by *mir-243*.

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Running head: *mir-243* modulates defenses against pathogens.

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Abstract

The interaction and communication between bacteria and their hosts modulate many aspects of animal physiology and behavior. Dauer entry as a response to chronic exposure to pathogenic bacteria in *Caenorhabditis elegans* is an example of a dramatic behavioral decision to survive. This response depends on the RNAi machinery suggesting the involvement of sRNAs as effectors. Interestingly, dauer formation occurs after two generations of interaction with moderate pathogenic bacteria of different species. Therefore, we sought to discover the identity of *C. elegans* RNAs involved in pathogenesis-induced diapause. Using transcriptomics and differential expression analysis of coding and non-coding long and small RNAs, we found that *mir-243-3p* is the only transcript upregulated in both animals exposed to either *P. aeruginosa* or *S.* Typhimurium for two generations. Phenotypical analysis of mutants showed that *mir-243* is required for dauer formation under pathogenesis but not under starvation. Moreover, DAF-16, a master regulator of key defensive processes in the animal and required for dauer formation was found to be necessary for *mir-243* expression. This work highlights the role of a small non-coding RNA in the intergenerational defensive processes against pathogenic bacteria and inter-kingdom communication.

Importance

Persistent infection of the bacterivore nematode *C. elegans* with bacteria such as *P. aeruginosa* and *S.* Typhimurium makes the worm diapause or hibernate. By doing this, the worm closes its mouth avoiding infection. This response takes two generations to be implemented. In this work we looked for genes that were turned on upon infection that could mediate the worm diapause triggered by pathogens. We identify *mir-243-3p* as the only transcript commonly upregulated when animals feed on *P. aeruginosa* and *S. enterica*. Moreover, we demonstrate that *mir-243-3p* is required for pathogen-induced dauer formation, a new function that has not been previously described for this miRNA. We find that the transcriptional activator DAF-16 is necessary for the expression of *mir-243* under pathogenesis. Here we establish a relationship between a small RNA and a behavioral change that ensures the survival of the progeny.

Introduction

C. elegans has a close ancestral relationship with bacteria (1), as it has naturally evolved exposed to microbes from the soil that can be their source of food or a threat (2, 3, 4). For decades in laboratory settings, *C. elegans* has fed on standardized bacteria, *E. coli* OP50. Our understanding of the nematode-bacteria relationship is evolving from a simple static organism/substrate pair to a dynamic model in which host and microbe´s roles change over the course of their association (5). The continuous encounter of *C. elegans* with bacteria has likely influenced its ability to recognize and defend from potential pathogens. *C. elegans*, when confronted with infectious microbes, can quickly evade them by modifying their olfactory preferences (6). This process is triggered by the detection of

specific molecules secreted by bacteria such as toxic pigments from *P. aeruginosa* PA14 (7); cyanide from *P. aeruginosa* PAO1 (8); and serrawettin W2, produced by *S. marcescens* (9). A recent report shows that the rapid avoidance response to *P. aeruginosa* PA14 involves DAF-7/TGF-β pathway in ASJ chemosensory neurons, synergistically with bacterial colonization and intestinal bloating (10). In contrast, a long-term strategy to ensure bacterial infection avoidance for long-term periods is dauer formation (11). This behavioral decision occurs after exposure to pathogens for two or more generations and is transmitted through the maternal germline and generates a transgenerational memory. Additionally, pathogen-induced diapause depends on the RNAi machinery suggesting the involvement of small RNAs (sRNAs) as mediators of this host-pathogen interaction.

Modulation of the host miRNAs is a molecular strategy exploited by bacteria to modify host cells, such as during *Salmonella* infection of mouse macrophages (12), human monocytes (12, 13), and zebrafish embryos (14). In *C. elegans*, the molecules signaling diapause entry in the second generation after pathogen exposure are unknown. This work focuses on the discovery of nematode's sRNAs involved in the response of *C. elegans* to chronic infection and leading to defensive dauer formation. Here we show that mature miRNA *mir-243-3p* is overexpressed in animals exposed to two unrelated pathogens and is needed to mount intergenerational diapause formation. We also show that transcription factors DAF-16, PQM-1 and CRH-2 are required for the expression of mature *mir-243*. PQM-1 and CRH-2 are also needed for dauer formation under pathogenesis. This work reveals a role for *mir-243* in the defense against pathogens and highlights the importance of small RNAs as mediators of long-term survival strategies.

Results

Global gene expression changes in intergenerational chronic exposure to pathogens

Behavioral decisions likely emerge from broad, complex gene expression changes. We aimed at revealing RNA profiles underlying the intergenerational diapause entry behavior (11). To this end we performed transcriptomic analysis of two generations of synchronized *C. elegans* in the L2 stage. Animals were grown on the two bacteria known to promote diapause *P. aeruginosa*, *S.* Typhimurium, and *E. coli* OP50, which does not trigger dauer formation. Sequencing was performed on both mRNA and small RNA libraries generated by separate methods (see Materials and methods). In this result section we will first refer to mRNAs. Differential gene expression of animals feeding on pathogenic bacteria was made in reference to non-pathogenic *E. coli* OP50 using DeSeq and EdgeR (**Dataset 1**). We considered differentially expressed genes those found to be statistically significant by either analysis (**Table S1** and **2**). Differentially expressed mRNA genes included coding and non-coding transcripts. We found that transcriptional changes in mRNAs (coding and non-coding) were larger in the first generation (F1) than in the second (F2) of animals fed with either pathogen (**Fig. 1A** and **B**). **Fig. 1C** to **E** illustrates the distribution of non-coding RNA expression in numbers (**C** and **D**) and types (**E**). Among non-coding transcripts we found piRNAs, 7k ncRNA, pseudogenes, tRNAs, lincRNAs, asRNAs, snoRNA, snRNA and rRNAs (**Fig. 1E**). The F1 generation was more abundant and diverse in differential expression than the F2s when feeding on either pathogen. This difference between generations could suggest that changes in the F1 are

most dramatic because they reflect changes in bacterial diet composition, as well as exposure to a pathogen. In this context, the subsequent generation may respond more specifically to each new bacterium.

Most mRNA genes were overexpressed or repressed in a generation or pathogen specific manner (**Table S3**). There were no shared upregulated mRNA genes in both pathogens and generations (**Fig. 1F**). However, a number of genes were commonly expressed in the F1, independently of whether animals were feeding on *P. aeruginosa* or *S.* Typhimurium. These genes grouped in Metabolic Processes by gene ontology (**Fig. S1A)** suggesting there is a common expression pattern that likely responds early to the change in diet from *E. coli* OP50 and it is not maintained after the F1 (see below). Interestingly, downregulated genes shared many more coincidences between pathogenic conditions (**Fig. 1G**). Three genes, *acdh-1*, *hpdp-1* and F54D5.12 were downregulated in both bacteria and generations. To distinguish the response to pathogens from changes in gene expression caused by the switch from *E. coli* OP50 to other bacteria we compared the expression changes in animals feeding *P. aeruginosa* PAO1 and *S.* Typhimurium in the F1 to those reported for *Comamonas. aquatica* (15). There were no coincidences in the upregulated genes (**Fig. S1B**), but several genes were repressed in animals feeding on all three bacteria (**Fig. S1C**), showing the change itself from *E. coli* OP50 triggers a characteristic common profile of gene expression. Additionally, these genes could be expressed in response to specific metabolites present in *C. aquatica*, *P. aeruginosa* and *S.* Typhimurium and not in *E. coli*.

We next examined whether genes differentially expressed in animals feeding on pathogens obeyed Gene Ontology (GO) patterns using the enrichment tool available from wormbase.org (16). We analyzed separately genes up and down regulated in each generation. There were no GO coincidences between animals feeding on either pathogen (**Fig. S2A**). This suggests that differential expression of mRNA genes considered in the enrichment is not likely to explain the phenotypical similarities triggered by both bacteria. *S.* Typhimurium could only be tested in the F1 because there was only one gene differentially expressed in the F2. We speculate that this is due to the high degree of genomic similarities between *Salmonella* and *E. coli* (17, 18). In *P. aeruginosa* both generations displayed GO enrichment, but the F2 was specifically enriched in genes involved in defense against biotic stress, showing that the defensive response is stronger against *P. aeruginosa* than to *S.* Typhimurium. GO enrichment of downregulated genes was larger in the F2 than in the F1 in animals feeding on either pathogen (**Fig. S2B)** showing a similar trend than those upregulated. Global analysis of mRNA gene expression shows that changes either individual or grouped are dissimilar for animals feeding on *S.* Typhimurium MST1 and *P. aeruginosa* PAO1. This suggests that changes giving rise to diapause formation are not due to mRNA change expression. To further understand the underlying molecular causes of pathogen induced dauer formation, we analyzed the sRNAs response to both bacteria.

sRNA expression changes in two generations of *C. elegans* exposed to bacterial pathogens

Small RNAs (sRNAs) are broad regulators of gene expression (19, 20, 21) and key candidate to modulate inter and transgenerational environmental adaptation (11, 22, 23). To identify sRNAs from *C. elegans* expressed in each bacterial condition we unbiasedly defined transcriptional peaks (TP, definition in Material and Methods). This method allows us to detect and precisely count expressed transcripts even if they were not previously annotated. We generated our own features based on the genomic coordinates of expression peaks. We evaluated if the features were novel (intergenic), overlapping, nested or perfectly matching database annotations to precisely measure expression of those fragments. Expressed TPs from *C. elegans* on all conditions included 95,5% of novel non-annotated sequences, in their vast majority located nested within coding genes (**Dataset 2**). Differentially expressed genes in all conditions included 6.2% of exact matches to known features, 77.8% of overlapping sequences and 16% of novel TPs located in intergenic regions (**Fig. 2A**). Differentially expressed sRNAs in worms feeding on pathogens include pre-miRNAs, mature miRNAs, TPs nested within rRNAs and 21-ur RNAs. Moreover, most novel TPs were nested in intronic or exonic segments of coding genes or overlapping 5' and 3´UTRs ends. There were also pseudogenic transcripts, tRNAs, and TPs in intergenic regions (**Fig. 2B** and **Table S4** and **5**). *mir-243*-3p along with another 11 TPs nested within the *rrn-3.1* ribosomal gene were upregulated in both generations in response to *P. aeruginosa* PAO1 and *S.* Typhimurium (**Fig. 2C** and **Table S6**). The pre*mir-70* and another 9 novel TPs were similarly downregulated in all conditions (**Fig. 2D** and **Table S6**). A number of TPs were specifically DE in response to MST1 or PAO1, or to both pathogens only one generation. For example, *mir-51* was upregulated in the F1 in PAO1, but *21ur-6043* was upregulated in both generations of animals feeding on PAO1 (**Table S6**). These results show that sRNA expression in the context of long-term pathogen exposure is mostly specific to one generation or to one pathogen. *mir-243-3p* however, is overexpressed in all conditions, suggesting it could be responding to the encounter with various bacteria. Furthermore, the specific response to PAO1 in the F2 was the largest in terms of numbers of TPs both up and downregulated (**Fig. 2E**). The following experiments were performed using *P. aeruginosa* PAO1.

mir-243 **is necessary for diapause formation under pathogenesis**

We first quantified the relative amounts of the mature *mir-243-3p* in each generation of animals feeding on *P. aeruginosa* PAO1 and compared it to those feeding on *E. coli* OP50. To that end, we grew animals on *P. aeruginosa* or *E. coli* for two generations, starting with L4 stage parentals (P0). F1 and F2 animals are collected as L2 for RNA extraction, as they were for the transcriptomic analysis. The relative fold of expression of miRNAs was quantified by RT-PCR. By this method, mature *mir-243-3p* appeared upregulated in the second generation feeding on *P. aeruginosa* PAO1. A mutation on *mir-243* (*n4759*) used as a control, completely abolished its expression (**Fig. 3A**). Then we explored whether *mir-243* has a role in diapause formation under pathogenesis by feeding mutant animals (*n4759)* with *P. aeruginosa* PAO1 for two generations. We also tested as a reference, animals with a deletion in *mir-253*, which was not differentially expressed under pathogenesis. This deletion also eliminates F44E7.15 expression, a non-coding RNA. *mir-243* and *mir-253/* F44E7.15 mutants were fed on *P. aeruginosa* PAO1 for two generations. The population growth and appearance of dauers was quantified in the F2 and compared to wild type animals. Growth on *P. aeruginosa* PAO1 was not affected by either

mutation (**Fig. 3B**) suggesting that these mutations do not increase susceptibility to pathogens. Interestingly, *mir-243* formed significantly less dauers than wild type or *mir-253*/F44E7.15 on *P. aeruginosa* PAO1 (**Fig. 3C**). We found that in *mir-253*/F44E7.15 pathogen-induced dauer is even more pronounced than in wild-type. Since none of the tested mutants were deficient in dauer formation under starvation (**Fig. 3D**), this result suggests that *mir-243* has a specific role in dauer formation under pathogenesis.

DAF-16 and other transcriptional activators regulate the expression of *mir-243* under pathogenesis.

The increase of mature *mir-243* in animals feeding on pathogens could be a result of transcriptional activation. To study whether this was the case, we quantified the fluorescence of a strain expressing *gfp* under the *mir-243* promoter (VT1474). We measured *gfp* in L2 animals in two generations of progenies of animals grown on pathogens and compared it with those grown on *E. coli* OP50. Animals feeding on pathogens overexpress *Pmir-243::gfp* compared to *E. coli* OP50 controls in both generations (**Fig. 4A**), suggesting that exposure to pathogens activates the transcription of *mir-243*. A number of transcription factors promote expression of their targets under stress and infection (24, 25, 26). In our paradigm, DAF-16/FOXO transcriptional activator localizes to the nucleus of animals exposed to *P. aeruginosa* PAO1 prior to diapause formation (11). We tested whether DAF-16 was necessary for *mir-243-3p* expression by extracting RNA from *daf-16* (allele) mutants and quantifying *mir-243-3p* in the F2 feeding on pathogens. **Fig. 4B** shows that *mir-243-3p* is not expressed in *daf-16* mutants.

As TFs-miRNAs likely work together in highly connected loops during development (27) we explored the role of other transcriptional regulators on *mir-243* expression. Under normal conditions, PQM-1 resides in the nucleus regulating the expression of DAF-16-associated elements (DAE) thus avoiding dauer formation (28). CRH-2 has been reported as a novel suppressor of the microRNA *let-7* (29) and could therefore regulate microRNAs more broadly. To study whether these transcriptional regulators are necessary for the expression of *mir-243* under pathogenesis we extracted RNA from *crh-2* and *pqm-1* mutants and quantified the expression of *mir-243-3p* in the F2 of worms fed with *P. aeruginosa*. **Fig. 4A** and **B** show that PQM-1 and CRH-2 are necessary for the over-expression of *mir-243-3p* when feeding on pathogens.

Because *mir-243* loss affects the ability of animals to form diapause under pathogenesis, we examined whether *crh-2* and *pqm-1* mutants failed to form dauers under infection but formed normal amounts of dauers under starvation. Additionally, we tested whether these mutants were able to grow on *E. coli* and *P. aeruginosa* to wild type extents. As a control we used a mutant of *elt-3* transcriptional activator with broad expression in the animal. *daf-16* mutation causes animals to be unable to form dauers (30) and could not be tested. All transcription factor mutants grew well on *E. coli* OP50 and to a similar extent as wild type animals on pathogenic bacteria (**Fig. 4C** and **D**). Interestingly, *crh-2* and *pqm-1* mutants were unable to diapause under pathogenesis after two generations on pathogens while *elt-3* animals formed normal amounts (**Fig. 4E**). All strains formed wild type numbers of dauers on starvation (**Fig. 4F**). This suggests that the role of these transcription

factors, like *mir-243* have on diapause formation is specifically in response to long-term pathogen exposure.

Consistently, *crh-2* and *pqm-1* promoters were overexpressed in pathogens compared to non-pathogenic conditions (**Fig. 4G** and **H**). Taken together these results suggest that an expression cascade started in the first generation triggered by the interaction with *P. aeruginosa* PAO1 includes CRH-2, PQM-1 and DAF-16 upstream of *mir-243* expression.

Discussion

Survival strategies to environmental challenges rely on the genetic plasticity of organisms. In this work, we dissect the transcriptomic differences and similarities between worms feeding on three different bacteria. Two of them, *P. aeruginosa* PAO1 and *S.* Typhimurium MST1 elicit dauer entry as a behavioral defense in the second generation (11). Differential gene expression analysis allowed us to identify *mir-243-3p* as a novel sRNA upregulated in animals fed on *P. aeruginosa* PAO1 and *S.* Typhimurium for two generations. Mutant animals of *mir-243* do not form the dauer larvae as a defensive mechanism against bacterial pathogens in the second generation despite not being dauer defective under starvation. Moreover, we identified transcription factors CRH-2, PQM-1, and DAF-16 as required for *mir-243* expression. Additionally, CRH-2 and PQM-1 are necessary for diapause formation under pathogenesis.

Most transcriptional changes are unique to the encounter with each pathogen

Gene expression is highly variable and dependent on environmental and physiological factors. *C. elegans* transcriptional profile is modified when animals are exposed to new bacterial food sources. These can be non-pathogenic such as *Bacillus subtilis* (31) or pathogenic such as *Shigella flexneri* (32), *P. aeruginosa* PA14 and *Staphylococcus aureus* NCTC8325 (33) (34). Environmental changes such as increased growth temperature can trigger gene expression associated with defensive responses and decreased expression of genes associated with metabolism (35); (31). ncRNA molecules enriched in these conditions indicate their function in a rapid adaptive response (36). The condition-specific transcriptomic variations can be transmitted to subsequent generations (37, 38, 39), suggesting that environmental changes promote phenotypic changes mediated by transcriptomic alterations that can be inherited to the progeny.

In this work, we discovered transcriptional changes that could explain the defensive decision to enter the dauer program as a response to pathogenesis (11). We notice that two different bacterial species trigger the same behavioral response in *C. elegans* but display few underlying transcriptomic similarities. We think that this could be an indicator of a very finely-tuned communication between bacteria and host. Clustering of genes by function (16) revealed large differences between animals fed the two bacteria. This could indicate that a particular behavior can be triggered by dissimilar patterns of expression, or that changes in a few genes can cause the behavioral switch. The upregulation of *mir-243* was the only coincidence among animals fed on pathogens. Additionally, *mir-243* is key for dauer formation due to pathogenesis. Although it is not yet clear what is the possible

mechanism by which this miRNA acts, we speculate that *mir-243* could be regulating a series of genes downstream that together signal the pathogenic state or execute the dauer entry program. *mir-243* acts as a siRNA: is recognized by RDE-1, which guides the miRNA towards a multi-protein complex for the recognition of targets and the destabilization of the mRNA molecule (40). Interestingly, RDE-1 has been shown, along with other RNAi effectors, to be required for dauer formation under pathogenesis (11). *mir-243* has shown to have perfect complementarity to its described target (40). This infrequent mechanism of silencing is much stronger than that normally triggered by a miRNA (40, 41). This could shed light on the reason why the loss of *mir-243* has a marked effect on a developmental and behavioral change such as diapause formation.

Non-coding RNAs in pathogenesis

Other works have explored the sRNA transcriptomic response to infection. For example, bacterial infection conditions affect piRNA biogenesis, which has been suggested in this context to increase animal fitness (42). The expression of *tts-1*, a lncRNA was significantly overexpressed in worm infected with *Microbacterium nematophilum* (43). Interestingly, the upregulation of *tts-1* have been associated with dauer entry by starvation in long-lived *daf-2* strain (44) and in larvae L1 (45). In this work we did not find differentially expressed genes whose mutations have been described before as conducing to abnormal dauer formation (*Daf*). This suggests at least two things, the expression of *Daf* genes does not necessarily change at the level of mRNAs, or that the dauer program under pathogenesis is molecularly different from starvation-induced diapause. *mir-243* for example is not required for dauer formation under starvation, but is essential for diapause under pathogenesis. This is coherent with the finding that most miRNAs are not essential for the development and survival of *C. elegans* perhaps because they are redundant (46).

microRNAs have previously been involved in the innate immune response of *C. elegans* against infection with bacterial pathogens. The *let-7* family members such as *miR-48*, *miR-84* and *miR-241* regulate developmental timing signals through the ATF-7 transcription factor, controlling cell proliferation by pathogenic bacteria (47, 48). In response to pathogens infection, *let-7* activity in immune response modulation occurs in the intestine, via regulation of SDZ-24-mediated signaling (49). Other candidate targets of *let-7* and *let-7* family microRNAs may include components of the PMK-1/p38 innate immune pathway (47). Likewise, *mir-67* mutants exhibited reduced pathogen avoidance behavior, apparently from the dysregulation of *mir-67* target, *sax-7* (50, 41). Changes in expression of miRNAs have also been observed in nematodes infected with eukaryotes such as the yeast *C. albicans* (51). *mir-70* or *mir-251/mir-252* mutants have moderate enhanced survival to infections with *P. aeruginosa* PA14 indicating that there is a negative regulation of the immune response by these three miRNAs (52). Interestingly, in our experiments, *mir-70* was systematically downregulated in animals feeding on both bacteria. This supports the idea that *mir-70* is a negative regulator of the innate immune response in *C. elegans*. Taken together, the previous findings, along with ours, states that that the response of *C. elegans* to pathogens is accompanied by dynamic changes in the activity of miRNAs.

Transcription factors involved in diapause formation as a defensive strategy

A number of transcription factors such as DAF-16 activate transcription of their targets under stress and infection. DAF-16 regulates genes with roles in defense against pathogenic bacteria such as *P. aeruginosa* PA14 (53), *S. enterica* strain 1344, *Yersinia pestis* strain KIM5, and *Staphylococcus aureus* MSSA476 (54). In the pathogen-induced dauer formation paradigm, DAF-16 localizes to the nucleus of animals exposed to *P. aeruginosa* PAO1 prior to diapause formation (11). In this work, we show that *mir-243* expression requires intact DAF-16. PQM-1 affects the expression of DAF-16 in the nucleus (28) and is needed for *mir-243* expression under pathogenesis. Interestingly, CRH-2, which has been proposed to regulate *let-7* miRNA transcription (55, 29), is also necessary for *mir-243* expression. Both proteins are overexpressed in the F2 of animals feeding on pathogens. Consistently, both CRH-2 and PQM-1 loss impair diapause formation under pathogenesis. In this work, we found that *mir-243* is involved in the response of *C. elegans* to infection with strains of *P. aeruginosa* or *S.* Typhimurium. We show that a mature form of *mir-243* is expressed in response to pathogenesis and required for dauer formation as a behavioral response. This means that changes in a single microRNA can have similar behavioral outputs, like dauer formation, but distinct molecular cascades to achieve it. Moreover, DAF-16, PQM-1 and CRH-2 transcription factors are needed for proper *mir-243* expression and subsequent dauer formation. Likely this is a multistep process that involves the establishment of the pathogenic state through bacterial and nematode transcriptional profiles and the execution of the behavioral decision after two generations. In this process miRNAs and other sRNAs could be playing important regulatory roles required for the phenotypic and transgenerational response in the animal.

Materials and Methods

C. elegans and bacterial growth

Wild type, mutant and transgenic *C. elegans* strains were grown at 20°C as previously described (56). All nematode strains were grown on *Escherichia coli* OP50-1 (resistant to streptomycin) before pathogen exposure. *S. enterica* serovar Typhimurium MST1 (ATCC 140828) and *P. aeruginosa* PAO1 (ATCC 15692) were used for infection protocols. All bacteria were grown overnight on Luria-Bertani (LB) plates at 37ºC from glycerol stocks. The next morning a large amount of the bacterial lawn is inoculated in LB broth and grown for 6 hours at 250 rpm and at 37ºC. 3 mL of the resulting bacterial culture is seeded onto 90 mm NGM plates and allowed to dry for 36 hours before worms are placed on them.

C. elegans strains

We used the following strains of the *Caenorhabditis* Genetics Center (CGC): Wild type (N2), MT15454 [*mir-243*(*n4759*)], MT16060 [*mir-253*(*nDf64*)], DR27 [*daf-16*(*m27*)], VC3149 [*crh-2* (*gk3293*)], RB711 [*pqm-1* (*ok485*)], VC143 [*elt-3* (*gk121*)], and transgenic strains VT1474 [*Pmir-243::gfp* (*unc-119* (*ed3*) III; *maIs177*)], OP201 [*unc-119* (*tm4063)* III; wgls201 (*pqm-1::TY1::EGFP*)], BC13136 [*crh-2* C27D5.4a*::gfp (dpy-5* (*e907*); sEx13136)], BC14266 [*dpy-5* (*e907*); sEx14266 (rCesF35E8.8::*GFP* + pCeH361)].

Hypochlorite treatment

To synchronize *C. elegans* and/or to obtain pure embryos, we prepared a 5% hypochlorite solution containing 20 mL of 1 M NaOH, 30 mL NaClO and 50 mL H_2O in 100 mL final volume. Plates with mostly gravid adults were washed with 1 mL of M9 (KH_2PO_4 3 g, Na_2HPO_4 6 g, NaCl 5 g, 1 M $MgSO_4$, H2O up to 1 L) and transferred to an Eppendorf tube. The volume collected was centrifuged at 394 x g. 1 mL of the hypochlorite solution was added to the pellet. After 5 minutes of vigorous vortex, the tube was centrifuged at 394 x g for 2 minutes. The pellet was washed with 1 mL of M9 solution and centrifuged at 394 x g and 2 minutes to discard the supernatant. The resulting pellet contains an embryo concentrate.

Quantification of population and dauer larvae

Dauer formation on pathogens. Entire worm populations on each plate were collected in 1 mL of M9. This initial stock was diluted 1:10 in M9. 10 µL of this 1:10 dilution was used to count the total population of worms under a Nikon SMZ745 stereomicroscope. To quantify the number of dauers in each population, the initial stock was diluted 1:10 in a 1% SDS solution and maintained in constant agitation for 20 minutes (57). To count the number of total animals and dauers, 10 µL of this last dilution was placed in a glass slide under the stereomicroscope. Each condition was scored 3 times (triplicated of one technical replica) and dauers were plotted as a percentage of the total populations of animals.

C. elegans growth in pathogenic bacteria

Five L4 (P0) wild type worms or mutants (grown in *E. coli* OP50) were picked and transferred to a 90 mm diameter plate seeded with 3 mL of *P. aeruginosa* PAO1, *S.* Typhimurium MST1 or *E. coli* OP50 control bacteria. In all cases, the bacterial lawn covered the plate. After 24 hours the F1 embryos were obtained by hypochlorite treatment. All obtained embryos were placed on a new plate with *P. aeruginosa* PAO1, *S.* Typhimurium MST1 or *E. coli* OP50. Animals were allowed to grow for 24 hours until they reached the L2 stage. The total number of worms in the population and the percentage of dauer were quantified.

To obtain the F2, five L4 larvae were transferred from *E. coli* OP50 to a 90 mm diameter plate with 3 mL of *P. aeruginosa* PAO1 or *S.* Typhimurium MST1. After eight days the total number of worms and dauer larvae were quantified. The number of bacteria seeded allowed animals to be well fed for the length of the experiment. In the case worms starved, we discarded that experiment. Each assay was performed in three independent experiments (technical replicates) generating a biological replica. A total of three biological replicates were considered for each analysis.

Dual RNA Seq

Sample preparation

Wild type *C. elegans* were cultured on 60 mm diameter Petri dishes with NGM media seeded with 500 µL of *E. coli* OP50 and maintained at 20°C. After three days, mixed stage animals were treated with bleaching solution and embryos were deposited in new dishes. 48 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, *S.* Typhimurium MST1 or *P. aeruginosa* PAO1. In all cases, the bacterial lawn covered the plate. Worms were allowed to grow at 20°C for 24 hours. After that time, all animals on the plate were subject to hypochlorite treatment. F1 embryos were collected in 1 mL of M9 and centrifuged at 394 x g. The embryos obtained were placed on a 90 mm new plate with 3 mL of bacteria. After 24 hours, animals were collected with M9 for total RNA extraction. Worms on other 3 plates were allowed to grow for another 48 hours until the F1 was gravid. F2 progenies were collected in the same way as the F1 and placed on a separate plate with the same species of bacteria. Animals were collected for RNA extraction 24 hours later. Each condition was performed in triplicates obtaining a total of 18 samples (F1 and F2 in *E. coli* OP50, *P. aeruginosa* PAO1 or *S.* Typhimurium MST1).

RNA extraction

RNA extraction of colonizing bacteria and C. elegans. Worms were washed off the plates with 1mL of M9, centrifuged at 394 x g for 2 min and resuspended at least 5 times with M9. RNA purification was performed using TRIzol (Life Technologies) following manufacturer's instructions. For RNA extraction, samples were mechanically lysed by vortexing with 4 mm steel beads for no more than five minutes. Each condition was performed in triplicates. Several biological replicas were mixed to reach the required concentration of 1 µg/µl of total RNA.

mRNA library preparation and sequencing. Total RNA was isolated from synchronized F1 and F2 *C. elegans* populations feeding on non-pathogenic *E. coli* OP50, and pathogens *P. aeruginosa* PAO1 and *S.* Typhimurium MST1 as explained above. After DNase I digestion with DNAse I (Invitrogen™), RNA concentration was measured using Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies). The integrity of RNA was determined on the Agilent 2100 Bioanalyzer (Agilent Technologies). mRNA libraries were prepared with the IlluminaTruSeq™ RNA sample preparation kit (Illumina) according to the manufacturer's protocol. The quality and size distribution of the libraries were evaluated with the Agilent 2100 Bioanalyzer using a DNA 1000 chip (Agilent Technologies) and quantified using the KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems) on the Step One Plus Real-Time PCR System (Applied Biosystems).

The *C. elegans* mRNAs libraries were sequenced using the HiSeq Illumina platform (BGI) with paired-end sequencing (2x 100 bp, BGI). *C. elegans* and bacterial small RNA were sequenced using the Mi-Seq Illumina platform at the Center for Genomics and Bioinformatics (CGB), Universidad Mayor.

sRNA library construction and sequencing. Samples were prepared and sequenced at CGB. Libraries were constructed with the TruSeq sRNA Sample Preparation Kit (Illumina), according to the manufacturer´s instructions. For quality control, cDNA libraries were run on a high sensitivity DNA chip using the Agilent 2100 Bioanalyzer (Agilent Technologies), according to the manufacturer's instructions. An agarose gel library size selection was performed to recover RNAs shorter than 200 nucleotides. The library was quantified using a high sensitivity DNA chip. The sRNA libraries were sequenced in an Illumina MiSeq sequencer using the MiSeq Reagent v2 50-cycle kit with single-end sequencing (1 x 36 bp). For each biological condition, three libraries were constructed from biological replicates.

Bioinformatic analysis

mRNA transcriptomics of C. elegans

Data pre-processing and quality control. Trimming was made with Trimmomatic v. 0.36 (58). Reads with a quality score (Phred score) less than 35 and read length less than 36 were removed.

Mapping and read count. Reads were aligned using Tophat (59) with default parameters. Reads were quantified using HTSeq count (60).

Differential expression was determined using EdgeR (61) and DeSeq (62). Differentially expressed genes were defined as those with adjusted p-value (padj) <0.05 by either method.

Enrichment analysis: Gene Ontology (GO) analysis was performed using the enrichment tool in wormbase (63).

Small RNAs transcriptomics of C. elegans

Data pre-processing and quality control. Quality visualization was made with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Illumina small 3' adaptors were removed with Cutadapt version 1.13 (64). Reads with an average quality over four bases lower than 30, as well as reads shorter than 17 bp were discarded with trimmomatics (65).

Mapping. For each sample, the reads were aligned against the *C. elegans* genome PRJNA13758 WS267 using bowtie2 version 2.2.6 (66). We chose a 17 bp seed length, which was the length of the shorter read. The seed interval was calculated as f(x) = 0 + 2.5 * sqrt(x), where x is the read length (roughly two seeds per read as reads are 36 or smaller). For greater sensitivity, we set the number of mismatches allowed in a seed to 1. By default, 15% of ambiguous characters per read is allowed. A bam file was produced for each sample.

Defining features and counting reads. We developed a strategy to detect both known and unannotated transcripts from bacteria and *C. elegans*. For that, we chose to make a reference annotation based on observed expression peaks. Some reads overlap annotated genes, others show more than one peak in annotated regions, and many peaks are located

in unannotated regions. 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 (more than 10 per base) and that may correspond to direct transcription or fragments of a longer transcript. This was done by merging bam files from each sample using SAMTools version 0.1.19. Afterward we only kept features between 17 and 150 nucleotides with an average coverage of 10 or more reads by nucleotide. 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. For comparison with databases, we intersected TPs with reported annotations and classified them according to their genomic context.

Comparison with annotated genes. To see how TPs matched with annotated genes, we compared them to the Ensembl database, by intersecting the GTF file of *C. elegans (PRJNA13758.WS267)* with our custom GFF file, using BEDTools (67). Based on this result, we classified TPs as novel (in intergenic unannotated regions), nested or overlapping annotated features, and sense or antisense to a known feature.

Differential expression analysis between conditions. For each sample, read count was performed with featureCounts (68) from the Bioconductor Rsubread package with default parameters. Then, the count matrix was used to perform differential expression analysis in R (version 3.3.2) between worms fed with three different bacteria of each generation using DeSeq2 (69) version 1.4.5. The samples of animals feeding on pathogenic strains *P. aeruginosa* PAO1 and *S.* Typhimurium were compared with *E. coli* OP50. For each condition, the first and second generations were compared.

We conducted the previous analysis using a custom-made bash and R scripts, available at https://mlegue@bitbucket.org/mlegue/cele.git or git@bitbucket.org:mlegue/cele.git.

Quantification of differential expression *by RT-PCR*

Total RNA Extraction: 5 L4 (P0) worms were placed in 90 mm NGM plates seeded with *P. aeruginosa* PAO1. After 24 hours, the total population was collected with 1 mL of M9 and centrifuged at 394 x g for 2 minutes. The pellet was treated with hypochlorite solution (see above). All resulting F1 embryos were placed in new plate with *P. aeruginosa* PAO1 for 24 hours and collected as L2. Collection is done with M9 in an Eppendorf tube. Contents were centrifuged at 394 x g for 2 min. L2 pellet was washed 5 times with M9 to eliminate the most bacteria from the sample. The pellet of synchronized worms (L2) was used for RNA extraction.

F2 animals were obtained from the same L4 P0 fed with *P. aeruginosa* and used to generate the F1 as explained above. F1 embryos were placed in a new 90 mm plate with 3 mL of *P. aeruginosa* PAO1. After 72 hours, the total population was collected with 1 mL of M9 and centrifuged at 394 x g for 2 minutes. The pellet was treated with hypochlorite solution. F2 embryos were placed in a new plate with *P. aeruginosa* for 24 hours. The L2 worms were washed off the plate with M9 and centrifuged at 394 x g for 2 min. 5 washes with M9 were necessary to eliminate most bacteria from the sample. The pellet of synchronized worms (L2) was treated with 4 mm steel beads and 1 mL of RNA-Solv® Reagent (Omega Bio-

Tek). The mix was vortexed for 5 minutes. RNA extraction was performed according to manufacturer specifications. Total RNA concentration was quantified with Tecan's NanoQuant Plate™. Each condition was performed in biological triplicates.

cDNA Synthesis. Using the extracted total RNAs, cDNA synthesis was performed with the MIR-X miRNA First Strand Synthesis from TAKARA following manufacturer's specifications.

Real Time PCR (*qPCR*). The cDNA samples (concentration 200 ng/µL) were used as a template to perform qPCR with the primers ©QIAGEN Ce_miR-243_1 miScript Primer Assay (MS00019481). The qPCR was performed with the MIR-X miRNA qRT-PCR TB Green Kit from TAKARA. To calculate the relative fold of expression of *mir-243-3p* between generations and genotypes we used delta-delta Ct calculations according to the Reference Unit Mass method (70). This method was based on the comparative use of the test sample against a calibrator (U6). Values less than 1 indicated the negative expression relation with respect to the control and a Ratio greater than 1 indicated the times above the sample with respect to the control.

Quantification of *gfp* expression

Expression levels of *pqm-1* and *crh-2* genes and the promoter of *mir-243 were* quantified by using wild type animals expressing *gfp*. *gfp* was quantified in F1 and F2 L2 fed with *P. aeruginosa* PAO1 and *E. coli* OP50. Worms were taken individually with a mouth pipette and placed in a bed of agarose with levamisole (20 mM) to immobilize them. To quantify *gfp* expression, we photographed entire animals on a Nikon Eclipse Ni microscope in 40X objective at 1/320s (*mir-243::gfp*) and 1/10s (*pqm-1* and *crh-2*) exposure time, ISO 200/3200 speed (white light and Laser respectively) and focal length of 50 mm. For all markers, photos of entire animals were taken. GFP quantification was done considering the signal from the entire animal. Image analysis was performed using ImageJ. Prior to the analysis images are converted to 8-bit format and a threshold between 1 and 1.2.

Statistical analysis

Statistical analyzes were carried out using one- or two-way ANOVA with post-hoc tests. For the differential expression analysis, statistical significance was considered lower than p-value <0.05. All experiments were repeated at least three times using technical replicas in each.

Figure Legends

Fig. 1. Global analysis of differential mRNA gene expression of an intergenerational infection paradigm. **A-B**. Number and types of mRNA genes differentially expressed on *P.*

aeruginosa PAO1 (A) and S. Typhimurium MST1 (B) in two generations. **C-D.** Number of non-coding mRNA genes differentially expressed on *P. aeruginosa* PAO1 (C) and S. Typhimurium MST1 (D) in two generations. **E**. Type and abundance of non-coding RNAs on *P. aeruginosa* PAO1 and *S*. Typhimurium MST1. **F-G.** Venn diagram representation of shared and unique genes up (F) and down (G) regulated in each generation and on each pathogen.

Fig. 2. Global analysis of differential small RNA gene expression of an intergenerational infection paradigm **A** Genomic context of sRNA genes differentially expressed in pathogenic conditions. **B** Type and abundance of sRNA genes differentially expressed on *P. aeruginosa* PAO1 and S. Typhimurium MST1 in two generations **C-D**. Venn diagram representation of shared and unique genes over-expressed (C) and repressed (D) in each generation and on each pathogen. **E**. Number of sRNA genes differentially expressed on *P. aeruginosa* PAO1 and S. Typhimurium MST1 in two generations.

Fig. 3. Expression of mature *mir-243* and role on pathogen induced diapause. **A**. Quantification of *mir-243-3p* expression in animals feeding on *P. aeruginosa* PAO1 for two generations. **B-D**. Growth (B), dauer formation on pathogens (C) and dauer formation by starvation (D) of *mir-243* mutant animals.

Fig. 4. Transcriptional factors required for *mir-243* expression and pathogen-induced diapause formation. **A**. Quantification of *mir-243* promoter expression by GFP in animals feeding on *P. aeruginosa* PAO1 compared to *E. coli* OP50 and representative photos. **B**. Quantification by RT-PCR of *mir-243-3p* on wild type and *daf-16*, *pqm-1* and *crh-2* mutant animals. **C-E**. Growth on *E. coli* OP50 (C), *P. aeruginosa* PAO1 (D) and dauer formation (E) in the second generation of animals. **F**. Dauer formation on starvation of wild type and mutants of transcription factors. **G-H**. Quantification of expression of PQM-1 (G) and CRH-2 (H) by GFP expression on animals fed on pathogens.

Fig. S1. **A**. Enrichment by Gene Ontology of upregulated genes in the F1 in both pathogens. **B-C** Coincidences in upregulated (B) and downregulated (C) genes of animals feeding on *C. aquatica* with *P. aeruginosa* or *S*. Typhimurium for one generation after change from *E. coli* OP50.

Fig. S2. Enrichment by GO term of upregulated (A) and downregulated (B) in animals feeding on *P. aeruginosa* PAO1 and S. Typhimurium MST1 in two generations.

Table S1-2. mRNA coding and non-coding genes differentially expressed in pathogenic conditions. Upregulated (1) and downregulated (2) genes expressed in F1 and F2 of animals feeding on *P. aeruginosa* PAO1 or *S.* Typhimurium MST1.

Table S3. Pathogen specific and shared differentially expressed genes in each condition.

Table S4-5. Small RNAs differentially expressed in pathogenic conditions. Up and downregulated sRNAs in the F1 and F2 of animals feeding on *P. aeruginosa* PAO1 (4) or *S*. Typhimurium MST1(5).

Table S6. Pathogen specific and shared differentially expressed genes in each condition. (14) upregulated and (15) downregulated genes.

Dataset 1. Differential expression analysis of expressed mRNA genes in two generations of animals exposed to *P. aeruginosa* PAO1 and *S.* Typhimurium MST1 by DeSeq and EdgeR.

Dataset 2. Differential expression analysis and genomic context of expressed sRNA genes in two generations of animals exposed to *P. aeruginosa* PAO1 and *S.* Typhimurium MST1.

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Author contribution

Conceptualization: CG, ML, MFP, LV, FG and AC

Methodology: CG, ML, MFP, LV, FG and AC

Investigation: CG, ML, MFP, LV, FG and AC

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References

1. **Cabreiro, F, Gems, D**. 2013. Worms need microbes too: microbiota, health and aging in *Caenorhabditis elegans*. EMBO Mol Med **5**:1300–1310.
2. **Schulenburg, H, Félix, MA**. 2017. The Natural Biotic Environment of *Caenorhabditis elegans*. Genetics **206**:55–86.
3. **Félix, MA, Ashe, A, Piffaretti, J, Wu, G, Nuez, I, Bélicard, T, Jiang, Y, Zhao, G, Franz, CJ, Goldstein, LD, Sanroman, M, Miska, EA, Wang, D**. 2011. Natural and experimental infection of *Caenorhabditis* nematodes by novel viruses related to nodaviruses. PLoS Biol **9**:e1000586.
4. **Troemel, ER, Félix, MA, Whiteman, NK, Barrière, A, Ausubel, FM**. 2008. Microsporidia are natural intracellular parasites of the nematode *Caenorhabditis elegans*. PLoS Biol **6**:2736–2752.
5. **Gerbaba, TK, Green-Harrison, L, Buret, AG**. 2017. Modeling Host-Microbiome Interactions in *Caenorhabditis elegans*. J Nematol **49**:348–356.

6. **Zhang, Y, Lu, H, Bargmann, CI**. 2005. Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. Nature **438**:179–184.
7. **Cezairliyan, B, Vinayavekhin, N, Grenfell-Lee, D, Yuen, GJ, Saghatelian, A, Ausubel, FM**. 2013. Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. PLoS Pathog **9**:e1003101.
8. **Gallagher, LA, Manoil, C**. 2001. Pseudomonas aeruginosa PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J Bacteriol **183**:6207–6214.
9. **Pradel, E, Zhang, Y, Pujol, N, Matsuyama, T, Bargmann, CI, Ewbank, JJ**. 2007. Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by *Caenorhabditis elegans*. Proc Natl Acad Sci U S A **104**:2295–2300.
10. **Singh, J, Aballay, A**. 2019. Microbial Colonization Activates an Immune Fight-and-Flight Response via Neuroendocrine Signaling. Dev Cell **49**:89–99.e4.
11. **Palominos, MF, Verdugo, L, Gabaldon, C, Pollak, B, Ortíz-Severín, J, Varas, MA, Chávez, FP, Calixto, A**. 2017. Transgenerational Diapause as an Avoidance Strategy against Bacterial Pathogens in *Caenorhabditis elegans*. mBio **8**
12. **Schulte, LN, Eulalio, A, Mollenkopf, HJ, Reinhardt, R, Vogel, J**. 2011. Analysis of the host microRNA response to *Salmonella* uncovers the control of major cytokines by the *let-7* family. EMBO J **30**:1977–1989.
13. **Maudet, C, Mano, M, Eulalio, A**. 2014. MicroRNAs in the interaction between host and bacterial pathogens. FEBS Lett **588**:4140–4147.
14. **Ordas, A, Kanwal, Z, Lindenberg, V, Rougeot, J, Mink, M, Spaink, HP, Meijer, AH**. 2013. MicroRNA-146 function in the innate immune transcriptome response of zebrafish embryos to *Salmonella typhimurium* infection. BMC Genomics **14**:696.
15. **MacNeil, LT, Watson, E, Arda, HE, Zhu, LJ, Walhout, AJ**. 2013. Diet-induced developmental acceleration independent of TOR and insulin in *C. elegans*. Cell **153**:240–252.
16. **Angeles-Albores, D, Lee, RYN, Chan, J, Sternberg, PW**. 2018. Two new functions in the WormBase enrichment suite. microPublication Biology
17. **AbuOun, M, Suthers, PF, Jones, GI, Carter, BR, Saunders, MP, Maranas, CD, Woodward, MJ, Anjum, MF**. 2009. Genome scale reconstruction of a Salmonella metabolic model: comparison of similarity and differences with a commensal Escherichia coli strain. J Biol Chem **284**:29480–29488.
18. **McClelland, M, Sanderson, KE, Spieth, J, Clifton, SW, Latreille, P, Courtney, L, Porwollik, S, Ali, J, Dante, M, Du, F, Hou, S, Layman, D, Leonard, S, Nguyen, C, Scott, K, Holmes, A, Grewal, N, Mulvaney, E, Ryan, E, Sun, H, Florea, L, Miller, W, Stoneking, T, Nhan, M, Waterston, R, Wilson, RK**. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413**:852–856.
19. **Lee, RC, Feinbaum, RL, Ambros, V**. 1993. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to *lin-14*. Cell **75**:843–854.
20. **Reinhart, BJ, Slack, FJ, Basson, M, Pasquinelli, AE, Bettinger, JC, Rougvie, AE, Horvitz, HR, Ruvkun, G**. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature **403**:901–906.

21. **Bartel, DP**. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116**:281–297.
22. **Rechavi, O, Minevich, G, Hobert, O**. 2011. Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. Cell **147**:1248–1256.
23. **Rechavi, O, Houri-Ze'evi, L, Anava, S, Goh, WSS, Kerk, SY, Hannon, GJ, Hobert, O**. 2014. Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. Cell **158**:277–287.
24. **Yang, W, Dierking, K, Rosenstiel, PC, Schulenburg, H**. 2016. GATA transcription factor as a likely key regulator of the *Caenorhabditis elegans* innate immune response against gut pathogens. Zoology (Jena) **119**:244–253.
25. **Blackwell, TK, Steinbaugh, MJ, Hourihan, JM, Ewald, CY, Isik, M**. 2015. SKN-1/Nrf, stress responses, and aging in *Caenorhabditis elegans*. Free Radic Biol Med **88**:290–301.
26. **Rohlfing, AK, Miteva, Y, Hannenhalli, S, Lamitina, T**. 2010. Genetic and physiological activation of osmosensitive gene expression mimics transcriptional signatures of pathogen infection in *C. elegans*. PLoS One **5**:e9010.
27. **Martinez, NJ, Ow, MC, Barrasa, MI, Hammell, M, Sequerra, R, Doucette-Stamm, L, Roth, FP, Ambros, VR, Walhout, AJ**. 2008. A *C. elegans* genome-scale microRNA network contains composite feedback motifs with high flux capacity. Genes Dev **22**:2535–2549.
28. **Tepper, RG, Ashraf, J, Kaletsky, R, Kleemann, G, Murphy, CT, Bussemaker, HJ**. 2013. PQM-1 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and longevity. Cell **154**:676–690.
29. **Lall, S, Grün, D, Krek, A, Chen, K, Wang, YL, Dewey, CN, Sood, P, Colombo, T, Bray, N, Macmenamin, P, Kao, HL, Gunsalus, KC, Pachter, L, Piano, F, Rajewsky, N**. 2006. A genome-wide map of conserved microRNA targets in *C. elegans*. Curr Biol **16**:460–471.
30. **Gottlieb, S, Ruvkun, G**. 1994. daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in *Caenorhabditis elegans*. Genetics **137**:107–120.
31. **Gómez-Orte, E, Cornes, E, Zheleva, A, Sáenz-Narciso, B, de Toro, M, Iñiguez, M, López, R, San-Juan, JF, Ezcurra, B, Sacristán, B, Sánchez-Blanco, A, Cerón, J, Cabello, J**. 2018. Effect of the diet type and temperature on the *C. elegans* transcriptome. Oncotarget **9**:9556–9571.
32. **Somasiri, P, Behm, CA, Adamski, M, Wen, J, Verma, NK**. 2020. Transcriptional response of *Caenorhabditis elegans* when exposed to *Shigella flexneri*. Genomics **112**:774–781.
33. **Irazoqui, JE, Troemel, ER, Feinbaum, RL, Luhachack, LG, Cezairliyan, BO, Ausubel, FM**. 2010. Distinct pathogenesis and host responses during infection of *C. elegans* by *P. aeruginosa* and *S. aureus*. PLoS Pathog **6**:e1000982.
34. **Shivers, RP, Youngman, MJ, Kim, DH**. 2008. Transcriptional responses to pathogens in *Caenorhabditis elegans*. Curr Opin Microbiol **11**:251–256.
35. **Jovic, K, Grilli, J, Sterken, MG, Snoek, BL, Riksen, JAG, Allesina, S, Kammenga, JE**. 2019. Transcriptome resilience predicts thermotolerance in *Caenorhabditis elegans*. BMC Biol **17**:102.

36. **Çelen, İ, Doh, JH, Sabanayagam, CR**. 2018. Effects of liquid cultivation on gene expression and phenotype of *C. elegans*. BMC Genomics **19**:562.
37. **Daxinger, L, Whitelaw, E**. 2010. Transgenerational epigenetic inheritance: more questions than answers. Genome Res **20**:1623–1628.
38. **Jaenisch, R, Bird, A**. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet **33 Suppl**:245–254.
39. **Heard, E, Martienssen, RA**. 2014. Transgenerational epigenetic inheritance: myths and mechanisms. Cell **157**:95–109.
40. **Corrêa, RL, Steiner, FA, Berezikov, E, Ketting, RF**. 2010. MicroRNA-directed siRNA biogenesis in *Caenorhabditis elegans*. PLoS Genet **6**:e1000903.
41. **Ambros, V, Ruvkun, G**. 2018. Recent Molecular Genetic Explorations of *Caenorhabditis elegans* MicroRNAs. Genetics **209**:651–673.
42. **Belicard, T, Jareosettasin, P, Sarkies, P**. 2018. The piRNA pathway responds to environmental signals to establish intergenerational adaptation to stress. BMC biology **16**:1–14.
43. **O'Rourke, D, Baban, D, Demidova, M, Mott, R, Hodgkin, J**. 2006. Genomic clusters, putative pathogen recognition molecules, and antimicrobial genes are induced by infection of *C. elegans* with *M. nematophilum*. Genome research **16**:1005–1016.
44. **Jones, SJ, Riddle, DL, Pouzyrev, AT, Velculescu, VE, Hillier, L, Eddy, SR, Stricklin, SL, Baillie, DL, Waterston, R, Marra, MA**. 2001. Changes in gene expression associated with developmental arrest and longevity in *Caenorhabditis elegans*. Genome Res **11**:1346–1352.
45. **Halaschek-Wiener, J, Khattra, JS, McKay, S, Pouzyrev, A, Stott, JM, Yang, GS, Holt, RA, Jones, SJ, Marra, MA, Brooks-Wilson, AR, Riddle, DL**. 2005. Analysis of long-lived *C. elegans daf-2* mutants using serial analysis of gene expression. Genome Res **15**:603–615.
46. **Miska, EA, Alvarez-Saavedra, E, Abbott, AL, Lau, NC, Hellman, AB, McGonagle, SM, Bartel, DP, Ambros, VR, Horvitz, HR**. 2007. Most *Caenorhabditis elegans* microRNAs are individually not essential for development or viability. PLoS Genet **3**:e215.
47. **Ren, Z, Ambros, VR**. 2015. *Caenorhabditis elegans* microRNAs of the *let-7* family act in innate immune response circuits and confer robust developmental timing against pathogen stress. Proc Natl Acad Sci U S A **112**:E2366–75.
48. **Zhu, Z, Zhang, D, Lee, H, Jin, Y**. 2016. *Caenorhabditis elegans*: An important tool for dissecting microRNA functions. Biomed Genet Genom **1**:34–36.
49. **Zhi, L, Yu, Y, Li, X, Wang, D, Wang, D**. 2017. Molecular Control of Innate Immune Response to *Pseudomonas aeruginosa* Infection by Intestinal *let-7* in *Caenorhabditis elegans*. PLoS Pathog **13**:e1006152.
50. **Ma, H, Marti-Gutierrez, N, Park, SW, Wu, J, Lee, Y, Suzuki, K, Koski, A, Ji, D, Hayama, T, Ahmed, R, Darby, H, Van Dyken, C, Li, Y, Kang, E, Park, AR, Kim, D, Kim, ST, Gong, J, Gu, Y, Xu, X, Battaglia, D, Krieg, SA, Lee, DM, Wu, DH, Wolf, DP, Heitner, SB, Belmonte, JCI, Amato, P, Kim, JS, Kaul, S, Mitalipov, S**. 2017. Correction of a pathogenic gene mutation in human embryos. Nature **548**:413–419.

51. **Sun, L, Zhi, L, Shakoor, S, Liao, K, Wang, D**. 2016. microRNAs Involved in the Control of Innate Immunity in Candida Infected *Caenorhabditis elegans*. Sci Rep **6**:36036.
52. **Kudlow, BA, Zhang, L, Han, M**. 2012. Systematic analysis of tissue-restricted miRISCs reveals a broad role for microRNAs in suppressing basal activity of the *C. elegans* pathogen response. Molecular cell **46**:530–541.
53. **Troemel, ER, Chu, SW, Reinke, V, Lee, SS, Ausubel, FM, Kim, DH**. 2006. p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. PLoS Genet **2**:e183.
54. **Singh, V, Aballay, A**. 2009. Regulation of DAF-16-mediated Innate Immunity in *Caenorhabditis elegans*. J Biol Chem **284**:35580–35587.
55. **Großhans, H, Johnson, T, Reinert, KL, Gerstein, M, Slack, FJ**. 2005. The temporal patterning microRNA *let-7* regulates several transcription factors at the larval to adult transition in *C. elegans*. Developmental cell **8**:321–330.
56. **Brenner, S**. 1974. The genetics of *Caenorhabditis elegans*. Genetics **77**:71–94.
57. **Cassada, RC, Russell, RL**. 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev Biol **46**:326–342.
58. **Bolger, AM, Lohse, M, Usadel, B**. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**:2114–2120.
59. **Trapnell, C, Pachter, L, Salzberg, SL**. 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics **25**:1105–1111.
60. **Anders, S, Pyl, PT, Huber, W**. 2015. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics **31**:166–169.
61. **Robinson, MD, McCarthy, DJ, Smyth, GK**. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**:139–140.
62. **Anders, S, Huber, W**. 2010. Differential expression analysis for sequence count data. Genome Biol **11**:R106.
63. **Angeles-Albores, D, N Lee, RY, Chan, J, Sternberg, PW**. 2016. Tissue enrichment analysis for *C. elegans* genomics. BMC Bioinformatics **17**:366.
64. **Martin, M**. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. journal **17**:10–12.
65. **Bolger, AM, Lohse, M, Usadel, B**. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**:2114–2120.
66. **Langmead, B, Salzberg, SL**. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods **9**:357–359.
67. **Quinlan, AR, Hall, IM**. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics **26**:841–842.
68. **Liao, Y, Smyth, GK, Shi, W**. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **30**:923–930.
69. **Love, MI, Huber, W, Anders, S**. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol **15**:550.
70. **Guénin, S, Mauriat, M, Pelloux, J, Van Wuytswinkel, O, Bellini, C, Gutierrez, L**. 2009. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot **60**:487–493.

Figure 1

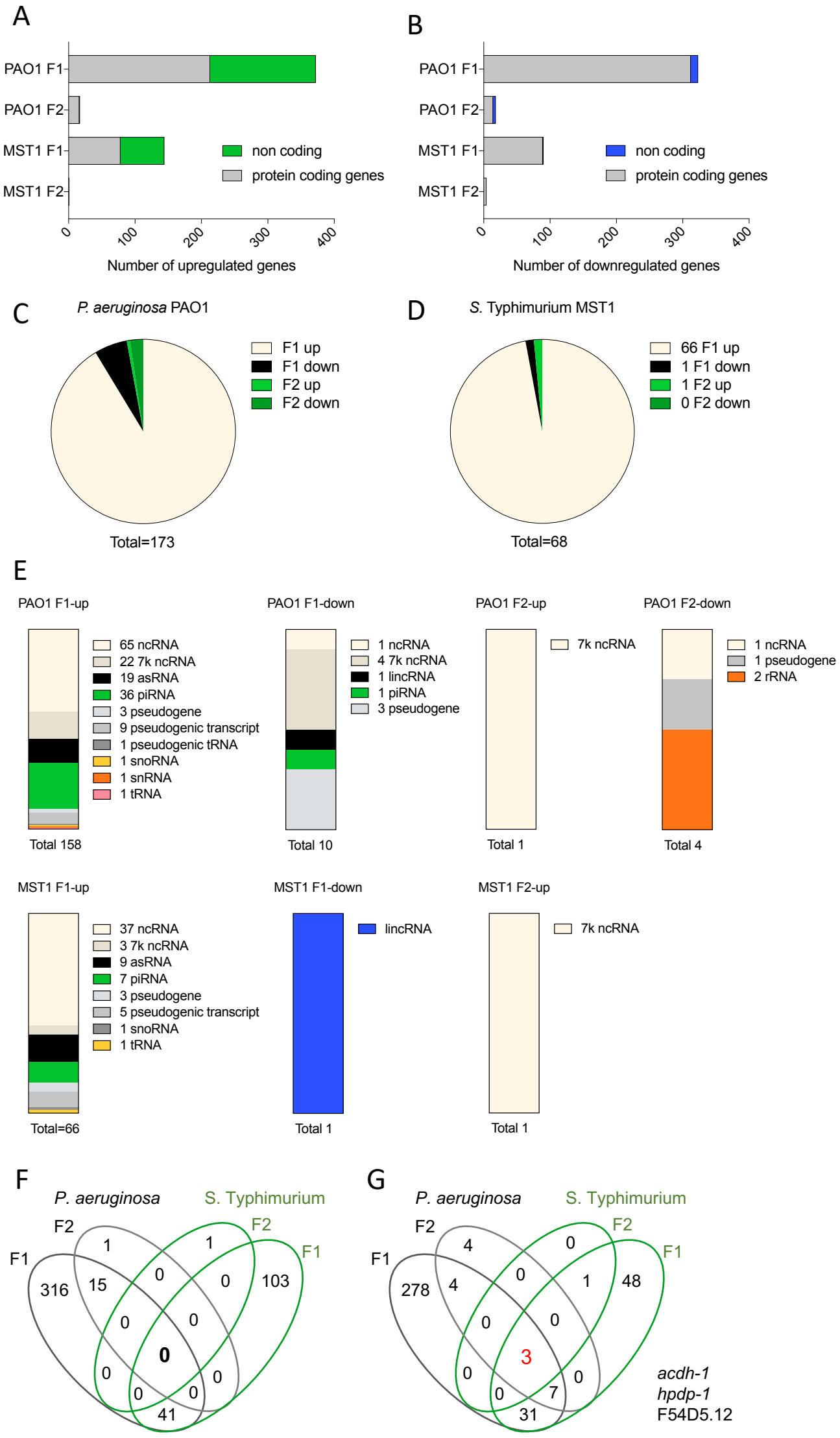

Fig. 1. Global analysis of differential mRNA gene expression of an intergenerational infection paradigm. **A-B**. Number and types of mRNA genes differentially expressed on *P. aeruginosa* PAO1 (A) and S. Typhimurium MST1 (B) in two generations. **C-D.** Number of non-coding mRNA genes differentially expressed on *P. aeruginosa* PAO1 (C) and S. Typhimurium MST1 (D) in two generations. **E**. Type and abundance of non-coding RNAs on *P. aeruginosa* PAO1 and *S.* Typhimurium MST1. **F-G.** Venn diagram representation of shared and unique genes up (F) and down (G) regulated in each generation and on each pathogen.

Figure 2

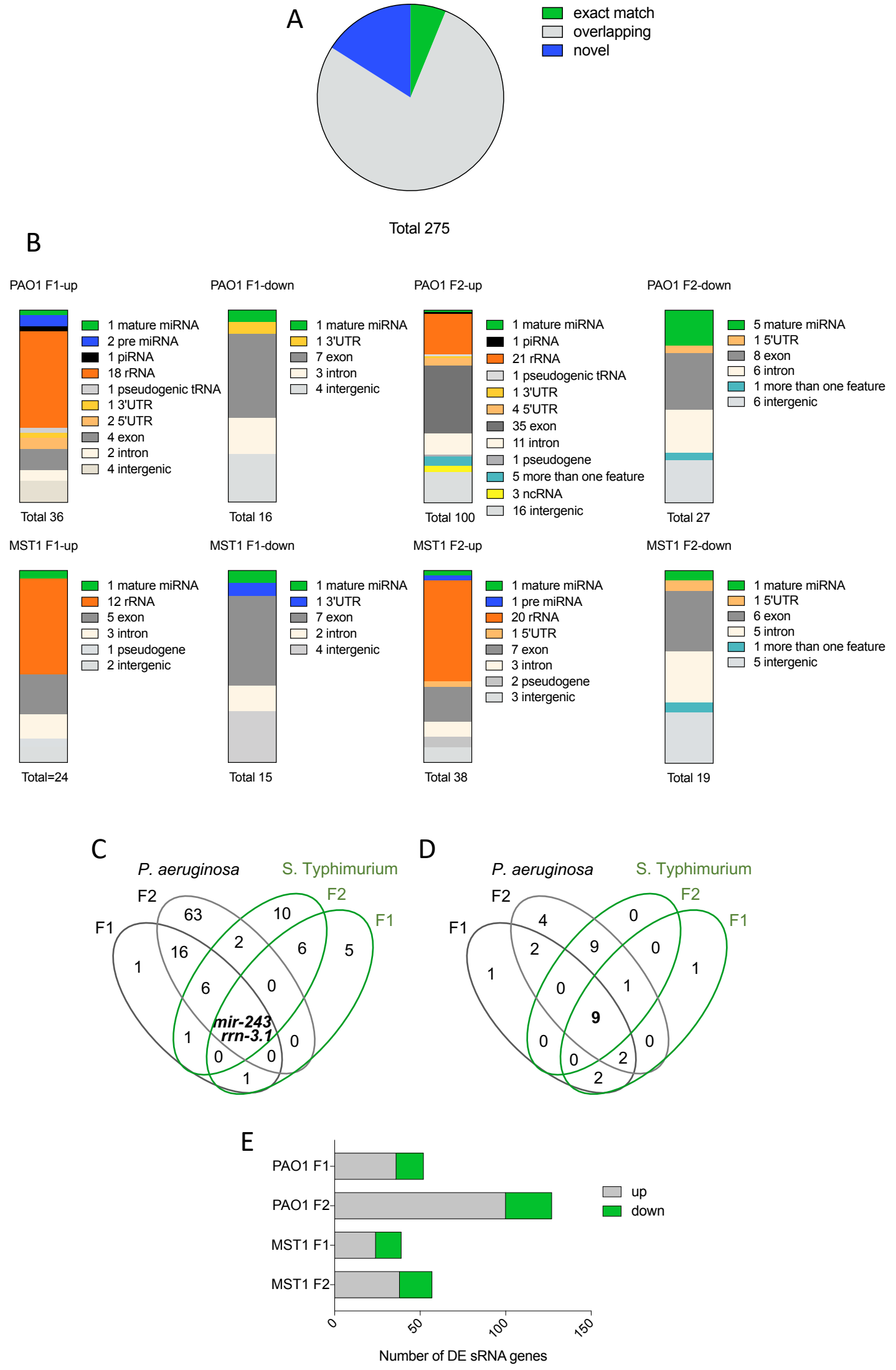

Fig. 2. Global analysis of differential small RNA gene expression of an intergenerational infection paradigm **A** Genomic context of sRNA genes differentially expressed in pathogenic conditions. **B** Type and abundance of sRNA genes differentially expressed on *P. aeruginosa* PAO1 and S. Typhimurium MST1 in two generations **C-D**. Venn diagram representation of shared and unique genes over-expressed (C) and repressed (D) in each generation and on each pathogen. **E**. Number of sRNA genes differentially expressed on *P. aeruginosa* PAO1 and S. Typhimurium MST1 in two generations.

Figure 3

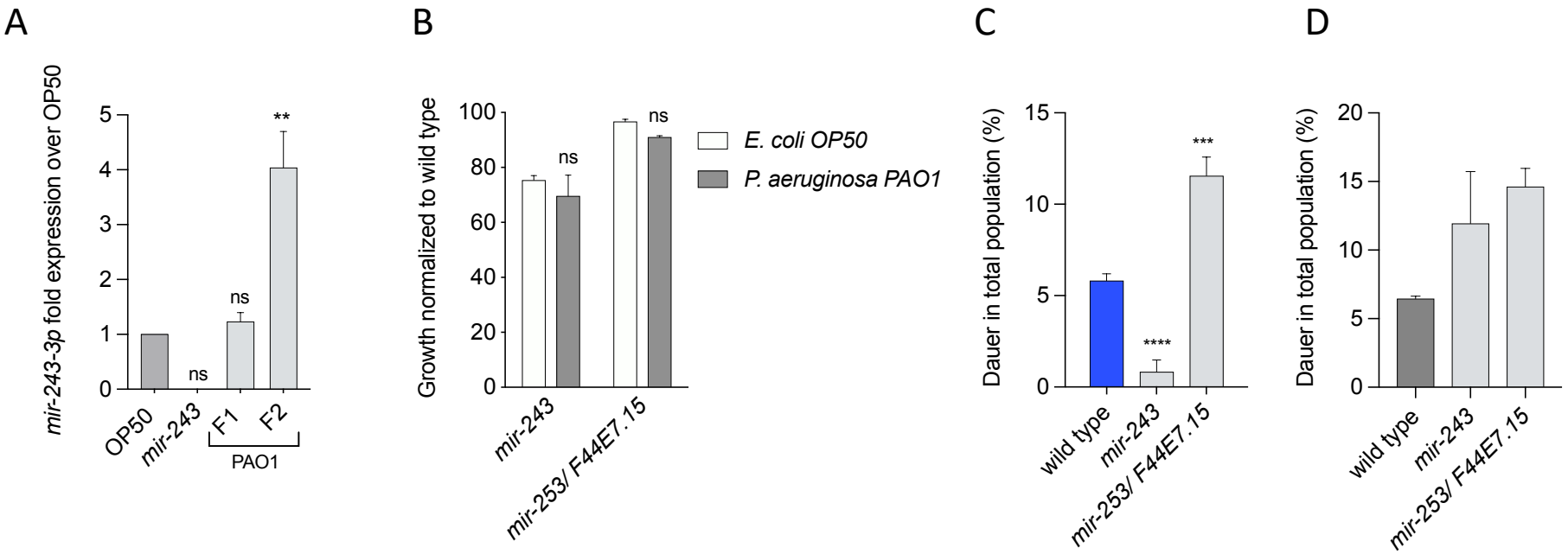

Fig. 3. Expression of mature *mir-243* and role on pathogen induced diapause. **A**. Quantification of *mir-243-3p* expression in animals feeding on *P. aeruginosa* PAO1 for two generations. **B-D** Growth (B), dauer formation on pathogens (C) and dauer formation by starvation (D) of *mir-243* mutant animals.

Figure 4

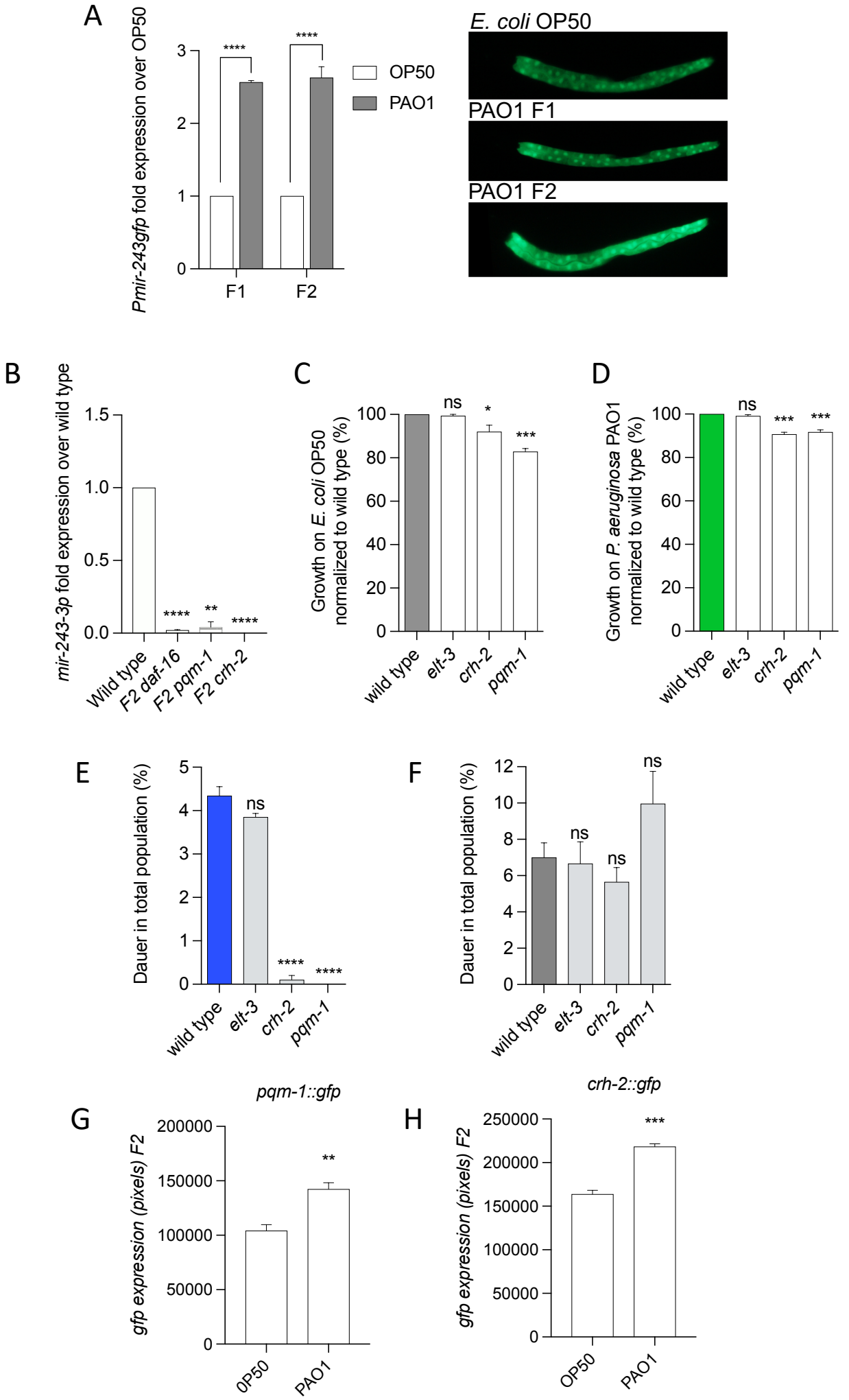

Fig. 4. Transcriptional factors required for *mir-243* expression and pathogen-induced diapause formation. **A**. Quantification of *mir-243* promoter expression by GFP in animals feeding on *P. aeruginosa* PAO1 compared to *E. coli* OP50 and representative photos. **B**. Quantification by RT-PCR of *mir-243-3p* on wild type and *daf-16*, *pqm-1* and *crh-2* mutant animals. **C-E**. Growth on *E. coli* OP50 (C), *P. aeruginosa* PAO1 (D) and dauer formation (E) in the second generation of animals. **F**. Dauer formation on starvation of wild type and mutants of transcription factors. **G-H**. Quantification of expression of PQM-1 (G) and CRH-2 (H) by GFP expression on animals fed on pathogens.