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Genome-wide association and environmental suppression of the mortal germline phenotype of wild *C. elegans*

Lise Frézal^{†,‡} , Marie Saglio[†], Gaotian Zhang , Luke Noble , Aurélien Richaud & Marie-Anne Félix^{*}

Abstract

The animal germline lineage needs to be maintained along generations. However, some *Caenorhabditis elegans* wild isolates display a mortal germline phenotype, leading to sterility after several generations at 25°C. Using a genome-wide association approach, we detect a significant peak on chromosome III around 5 Mb, confirmed by introgressions. Thus, a seemingly deleterious genotype is maintained at intermediate frequency in the species. Environmental rescue is a likely explanation, and indeed associated bacteria and microsporidia suppress the phenotype of wild isolates as well as mutants in small RNA inheritance (*nrde-2*) and histone modifications (*set-2*). *Escherichia coli* strains of the K-12 lineage suppress the phenotype compared to B strains. By shifting a wild strain from *E. coli* K-12 to *E. coli* B, we find that memory of the suppressing condition is maintained over several generations. Thus, the mortal germline phenotype of wild *C. elegans* is in part revealed by laboratory conditions and may represent variation in epigenetic inheritance and environmental interactions. This study also points to the importance of non-genetic memory in the face of environmental variation.

Keywords *C. elegans*; epigenetics; GWAS; microbiota; mortal germline

Subject Category Evolution & Ecology

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Introduction

The animal germline is an immortal cell lineage that persists through organismal generations. The nematode *Caenorhabditis elegans* displays a short generation time of about 3 days under laboratory conditions, allowing studies of the conditions for germline immortality. Indeed, Ahmed and Hodgkin screened for mutants of the N2 reference strain with a “mortal germline” (Mrt) phenotype, defined as a progressive onset of sterility across generations (Ahmed & Hodgkin, 2000; Smelick & Ahmed, 2005).

Among the genetic loci thus defined, a subset displayed a temperature-sensitive (ts) Mrt phenotype, even as null alleles (Sakaguchi *et al.*, 2014). Over the last 10 years, a number of loci were shown to display this ts-Mrt phenotype. The corresponding mutations so far affect nuclear small RNA pathways, small RNA amplification, and histone modifications in the germline, relating the ts-Mrt phenotype to transgenerational epigenetic inheritance (Buckley *et al.*, 2012; Robert *et al.*, 2014; Spracklin *et al.*, 2017; Weiser *et al.*, 2017; Manage *et al.*, 2020; Wan *et al.*, 2021).

Epigenetic inheritance of gene expression states operates in *C. elegans* through small RNAs and histone modifications (Frolows & Ashe, 2021; Grishok, 2021; Ketting & Cochella, 2021; Phillips & Updike, 2022; Quarato *et al.*, 2022; Seroussi *et al.*, 2022). Briefly, small RNAs bound to specific Argonaute proteins enter the nucleus and induce histone modifications such as repressive H3K9me3 or H3K27 methylation at the corresponding locus, thereby silencing gene expression (Gu *et al.*, 2012; Mao *et al.*, 2015). Small RNAs corresponding to germline-expressed genes are amplified in perinuclear germ granules (Phillips *et al.*, 2012; Ishidate *et al.*, 2018; Uebel *et al.*, 2018; Wan *et al.*, 2018, 2021), allowing for persistence of silencing for several generations. A subset of mutants in nuclear RNAi (such as *nrde-2*) or in germline nuclear Argonaute genes (such as *hrde-1*) display the ts-Mrt phenotype (Burton *et al.*, 2011; Buckley *et al.*, 2012). In addition, mutants in histone methyltransferases, such as *set-2/SET1* (encoding the histone H3K4me3 methyltransferase), display the ts-Mrt phenotype. A balance of histone modifications appears necessary to maintain germline pluripotency and DNA integrity (Katz *et al.*, 2009; Alvares *et al.*, 2014; Robert *et al.*, 2014, 2020; Herbet *et al.*, 2017; McMurchy *et al.*, 2017; Saltzman *et al.*, 2018). Thus, the ts-Mrt phenotype may be an indication of variation in gene-silencing mechanisms.

We previously reported that, surprisingly, multiple *C. elegans* wild isolates displayed the ts-Mrt phenotype (Frézal *et al.*, 2018). This phenomenon is interesting for two reasons: first, a sterility phenotype is an odd one to find in a wild population; second, this finding points to possible natural genetic variation in transgenerational epigenetic inheritance. In the first study, we focused on a genetic cross between two isolates of contrasting Mrt phenotype (MY10 and

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JU1395), which identified a causal deletion in the *set-24* gene underlying the strong Mrt phenotype of MY10. This gene encodes a SET-domain protein, likely involved in producing or reading post-translational histone modifications. The *set-24* deletion allele is rare across the species (Frézal *et al*, 2018). Therefore, its evolutionary importance is difficult to assess.

Saber *et al* recently reported on the frequency of the Mrt phenotype in wild *C. elegans* isolates and its low mutational variance using mutation accumulation lines. They suggested that the phenotype—or the underlying genetic polymorphisms—may be under balancing selection (Saber *et al*, 2022). We here asked (i) whether we could detect a common polymorphism underlying the Mrt phenotype of *C. elegans* wild isolates; and (ii) whether the phenotype could be suppressed by natural environmental conditions.

We first perform a genome-wide association of the Mrt phenotype on over 100 *C. elegans* wild isolates and detect a signal on chromosome III at a genomic location close to 5 Mb. This locus could be confirmed using introgression lines. To explain this common occurrence of a deleterious phenotype, we isolated natural populations of *C. elegans* with their associated microbes. The strains deprived of their natural microbiota exhibit an Mrt phenotype but show a strong rescue when reassociated with isolated bacteria or microsporidia. *E. coli* strains of the K-12 lineage also suppress the Mrt phenotype compared to B lineage strains. We report that the Mrt phenotype of *nrde-2*, *set-2*, and *set-24* mutants is also suppressed by bacterial associations, and is thus revealed by standard, simple laboratory conditions. Furthermore, we find that a wild *C. elegans* strain can keep the memory of its culture on a suppressing bacterial strain.

Results

Genome-wide association of the mortal germline phenotype

We measured the mortal germline (Mrt) phenotype of 132 world-wide *C. elegans* isolates at 25°C, using the assay in Frézal *et al* (2018). The phenotype was coded as a quantitative trait, which we call the Mrt-value, defined as the mean number of generations to sterility.

The 25°C Mrt phenotype was found to be common among *C. elegans* wild isolates (Fig 1). The population structure of *C. elegans* includes highly divergent strains from the Pacific region and a less structured population on the Eurasian, African, and American continents (Crombie *et al*, 2019; Lee *et al*, 2021). Strains in both groups were found to exhibit an Mrt phenotype (Figs 1A and EV1A; Table EV1). We did not find a correlation with the temperature at the time of isolation (Fig 1D).

In the panel of 132 strains, the mean Mrt-values of strains ranged from sterile at 2 generations to still fertile after 20 generations at 25°C, with a skewed distribution toward high values (Fig 1B). The variance among replicates was low for strong Mrt-values and high at intermediate trait values (Fig 1C). Given this distribution, we analyzed the data using generalized linear mixed models (cf. Material and Methods). Each Mrt assay started at a certain date constitutes an experimental block. The Mrt-values were obtained from different experimental blocks, including different experimenters and laboratories. A significant block effect was detected, which was taken into account in the analysis.

A genome-wide association study (GWAS) is a genetic mapping that uses the natural diversity of a panel of organisms of a given species to test for statistical independence between the allelic state of polymorphic markers and the phenotype of interest (Andersen & Rockman, 2022). A statistical association between the marker and the phenotype indicates that a polymorphism tightly linked to the marker in the data (i.e., in linkage disequilibrium with it) causes the variation in phenotype. For statistical reasons, GWAS can only detect polymorphisms that are at intermediate frequencies in the panel, that is, cases where both alleles occur at frequencies higher than 5%. We only used such polymorphisms in the GWAS (see Methods). We included data collected by Saber *et al* (2022) and used either the full strain set (Fig EV1B), or a restricted set (Fig 1E and F) without the divergent Pacific area strains (as defined by Crombie *et al*, 2019, and Lee *et al*, 2021, Table EV1). Using the restricted set of 113 strains, we found that variation in the number of generations to sterility was significantly associated with genetic variation in a region of chromosome III located around 5 Mb (Fig 1E). The larger strain set including divergent Pacific region strains also gave a significant peak on chromosome III at 5 Mb and in addition a significant association on chromosome X (Fig EV1B). The chromosome III association was stronger with the restricted set of isolates, in line with the fact that the additional strains are genetically divergent and practically isolated from the continental recombinant pool (Crombie *et al*, 2019; Lee *et al*, 2021). We further focused on this chromosome III association.

To diminish the multiple testing burden, the initial analysis in Fig 1E used a restricted set of markers, after pruning those that were in high linkage to each other. We further performed fine mapping in the chromosome III region using all SNPs (Dataset EV1). For SNPs with a high significance ($P < 10^{-4}$) in the fine mapping, the frequency of the Mrt-associated allele was comprised between 21 and 41% in our GWAS strain set (Dataset EV1); as an example, the Mrt allele of the associated SNP shown in Fig 1F (III:4677491) displayed a frequency of 29% in the restricted strain set. Over the global wild strain set with genotypes at CeNDR in 2020, these numbers were 17–58% and 39%, respectively. Note that SNPs were used as markers and that the causal polymorphism may be an insertion or deletion.

The alleles associated with the Mrt phenotype were carried by several haplotypes in the 4.5–6 Mb region, showing a history of both mutation and recombination (Fig EV2). A subset of Mrt-associated alleles, including that at III:4677491, are also present in divergent Pacific strain haplotypes (Dataset EV1; Fig EV2C for the example SNP); however, we note that not all candidate SNPs displayed this pattern, as some were specific to haplotypes on the “JU775” branch in Fig EV2C and absent in divergent Pacific isolates and JU1793 (Dataset EV1).

We further interrogated the geographic distribution of the relevant chromosome III haplotypes based on genotypes available at CeNDR (Cook *et al*, 2017). The “JU775” and “JU1793” chromosome III haplotypes (Fig EV2) and a genotype with reference N2 alleles were found to coexist in Europe, Africa, North America, and Oceania, and at a smaller geographical scale at all three of the heavily sampled locations in Richaud *et al* (2018) (Orsay, Santeuil, and Plougasnou) (Table EV2). In addition, using sampling locations in Barrière & Félix (2007) and Haber *et al* (2005), we found that a

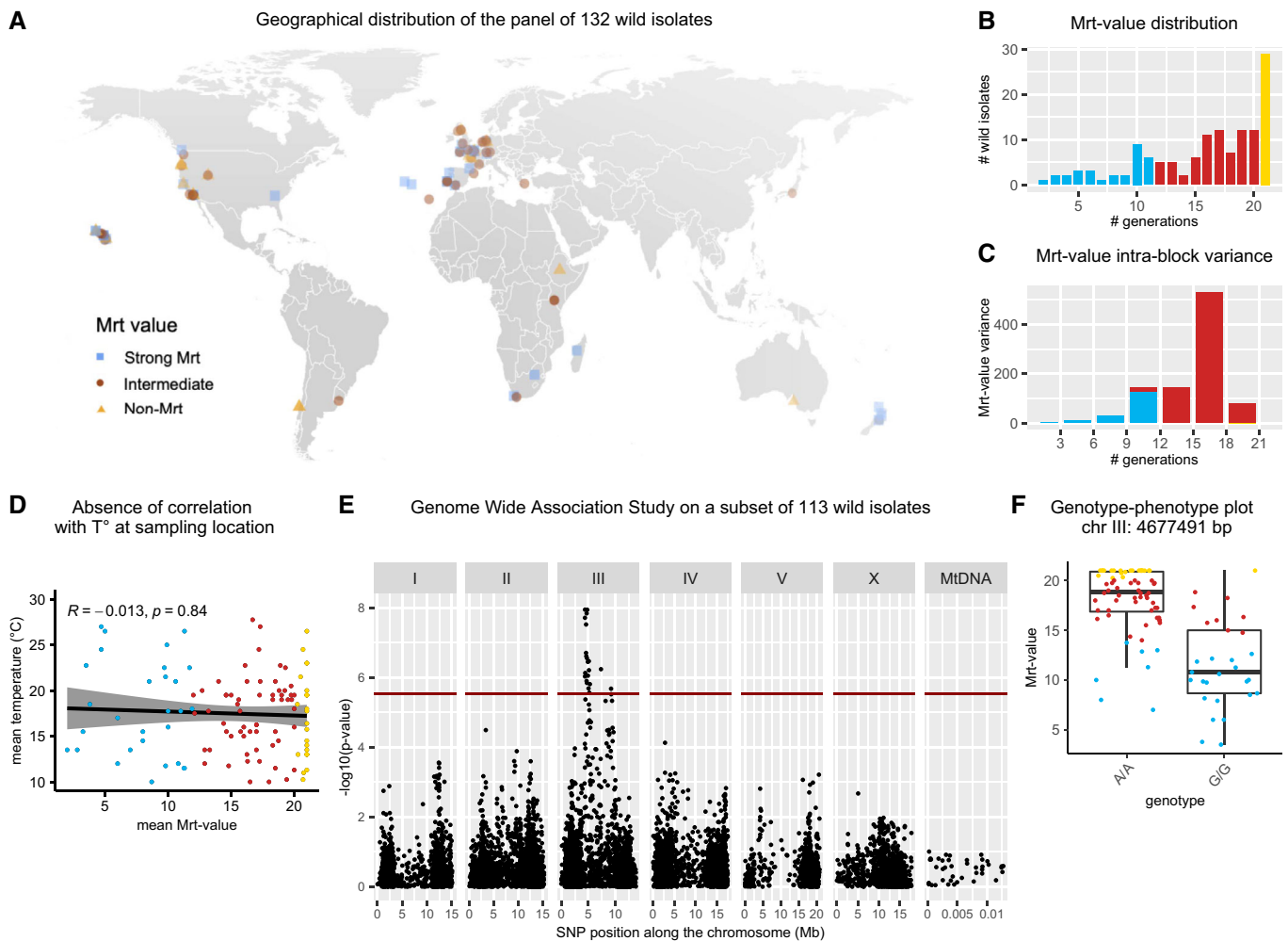


Figure 1. Distribution of the mortal germline (Mrt) phenotype in *C. elegans* wild isolates and genetic association with a locus on chromosome III.

- A Geographical distribution of the 132 *C. elegans* wild isolates, color-coded according to their Mrt phenotype. The same Mrt color code applies to all panels, with the blue color indicating strong Mrt strains (sterile before 12 generations), the red intermediate Mrt strains (sterile at 12–20 generations), and the gold color for the non-Mrt strains (that never became sterile). At locations with several tested strains, the superimposed symbols take a darker shade.
- B Phenotypic distribution of the Mrt phenotype of the 132 *C. elegans* wild isolates, assayed as in Frézal et al (2018) and expressed as the number of generations to sterility at 25°C, until generation 20. Fertility in this last generation is plotted as generation 21. The data are derived from 11 experimental blocks as shown in Fig EV1.
- C Intra-block variance of the Mrt phenotype of the 132 *C. elegans* wild isolates plotted as a function of the Mrt-value mean. The Mrt-values are binned in intervals of three generations.
- D Absence of correlation between the Mrt-value and temperature at the sampling location of the 132 *C. elegans* wild isolates. The graph shows the relationship between temperature of isolation and mean Mrt-value in number of generations, with the regression line in black and the 95% confidence interval in gray. The result of a Kendall correlation test is shown above the graph.
- E Genome-wide association using a subset of 113 *C. elegans* wild strains, excluding the divergent Pacific strains. The plot represents the significance value of the association between the tested single-nucleotide polymorphisms (SNPs) plotted along the six chromosomes and mitochondrial DNA (MtDNA), using a generalized linear mixed model. The red line indicates the significance threshold at $P < 0.05$, estimated using permutations. The genomic position is expressed in megabases (Mb). See Fig EV1B for GWAS including the divergent Pacific strains and Dataset EV1 for fine mapping with the full set of SNPs.
- F Genotype–phenotype plot for a highly associated SNP (III:4677491 bp; chosen to be consistent with the interval derived from the laboratory recombinants in Fig 2). The interquartile range is displayed as a box, the median as a horizontal line, and the whiskers represent 1.5× the interquartile range. Each dot represents one *C. elegans* wild isolate used in the association in panel E ($n = 113$), with its mean Mrt phenotype expressed in number of generations.

Source data are available online for this figure.

“JU775” and a reference chromosome III haplotype co-existed in at least 8/10 locations, while the two others appeared to only carry a reference allele (Table EV2). Overall, this shows that the causal polymorphism(s) is present at an intermediate frequency and is maintained as such in local and worldwide natural populations.

Introgression lines confirm the association with chromosome III

To test the causal effect of chromosome III, we produced chromosome introgression lines between parents of contrasted phenotypes. We chose JU775 as parent with an Mrt phenotype because this

isolate carries the chromosome III alleles associated in the GWAS analysis with the Mrt phenotype (Fig EV2). We introgressed chromosome III of JU775 into the N2 genetic background using repeated backcrosses associated with genotyping and tested the Mrt phenotype of parents and introgression lines.

The strains with the N2 genetic background could be propagated in the laboratory for over 20 generations at 25°C (Fig 2A—this included SX461 that carries a here irrelevant single-copy *gfp* transgene). By contrast, the strains with the JU775 genetic background were sterile before eight generations at 25°C. The strains carrying the JU775 chromosome III in the N2 genetic background, JU3194 and JU3195, showed a stronger Mrt phenotype compared to the non-Mrt parent (Fig 2A). The Mrt-values of the chromosome III introgression lines were intermediate between the parental values, indicating that polymorphisms on other chromosomes must explain an additional part of the difference between JU775 and N2.

We then screened for recombinants after further crossing the chromosome III introgressed line JU3395 to the N2 genetic background. The recombinant lines were assayed for the Mrt phenotype in several experimental blocks (Fig 2B). The first group of recombinants displayed a strong Mrt phenotype, comparable to that of the parent JU3395. This group is composed of the recombinants JU4309, JU4340, JU4341, and JU4313. These strains share JU775 alleles in a 4.66–6.49 Mb region on chromosome III (Fig 2B). Therefore, this region is sufficient to give a strong Mrt phenotype. This removed as candidate loci some highly associated alleles on the left side of the GWAS interval (in gray in Dataset EV1). A second group of recombinants that did not include this interval showed a mild Mrt phenotype and thus exhibited more variance, both across replicates of a given experiment, and across experiments (indicated as mild Mrt in Fig 2B). Note that the SX261 (N2 background) parent also showed variation among experimental blocks. Our focus was on the GWAS peak; thus, we did not investigate further whether a

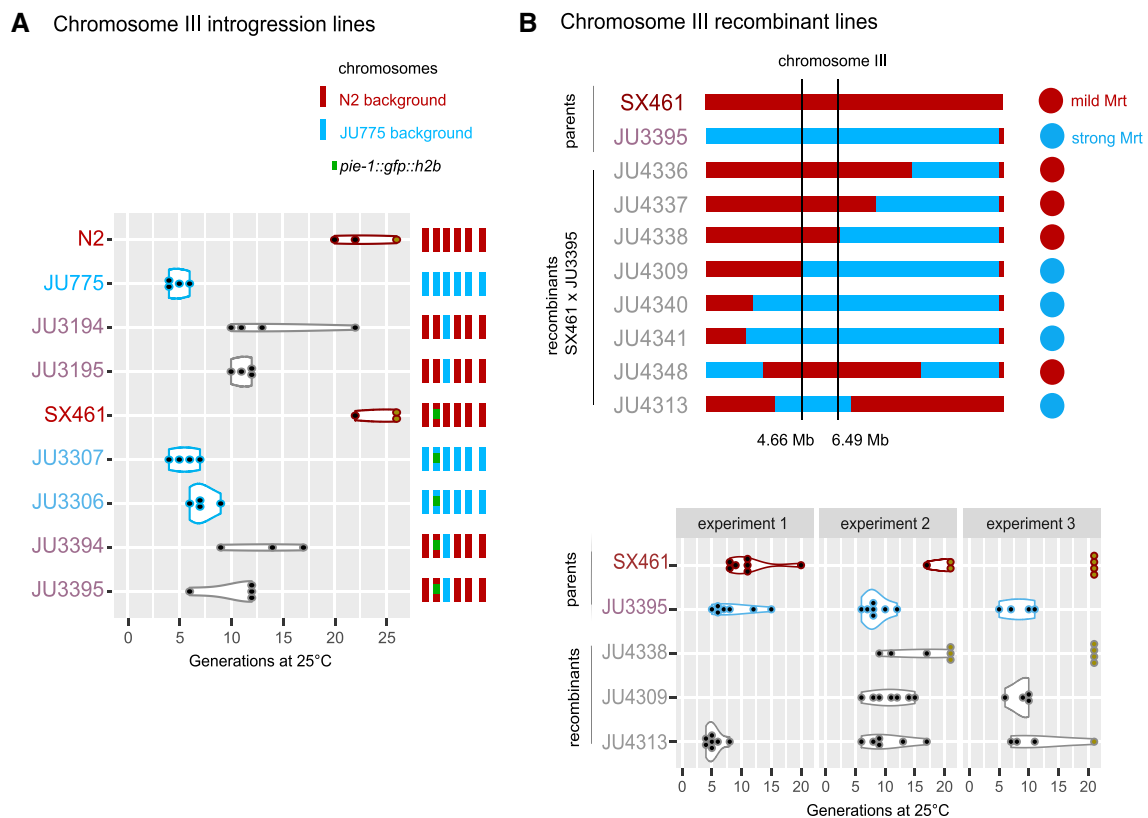


Figure 2. Laboratory crosses confirm the presence of a quantitative trait locus on chromosome III at ca. 5 Mb.

A Mrt phenotypes of the *C. elegans* chromosome III substitution lines. In red is indicated the N2 genetic background (non-Mrt), in light blue the JU775 genetic background (Mrt), and in violet the introgression lines. The two graphs are two replicate experiments without and with a *gfp* transgene, indicated as a green bar. Both chromosome III and the remaining background had a significant effect in an ANOVA on a Poisson generalized linear model (each at $P < 10^{-7}$).

B Mrt phenotypes of the *C. elegans* chromosome III recombinant lines. Top panel: Schematic of genotypes of the recombinants between JU3395 and SX461, with in blue JU775 alleles and in red N2 alleles. Phenotyping data from multiple experiments are in Source data and schematized on the right in a binary fashion with a blue circle showing a strong Mrt phenotype, and a red circle otherwise. Bottom panel: Mrt phenotyping of the parents and a subset of recombinant lines, in three independent experiments. The JU3395 parent and the JU4309 and JU4313 recombinants exhibit a strong Mrt phenotype, while the SX461 parent and the JU4338 recombinant have a mild and more variable Mrt phenotype.

Data information: The Mrt phenotype is expressed in number of generations to sterility. Dots represent biological replicates, all assayed in the same experiment if in the same graph. The dark gold color of the dots highlights replicates that remained fertile.

Source data are available online for this figure.

second locus present in JU775 on the right of chromosome III may have a smaller effect.

Altogether, these results show that a polymorphism located between 4.66 and 6.49 Mb on chromosome III strongly affects the Mrt phenotype in a cross between N2 and JU775. This genomic position is in accord with the GWAS peak. Statistical association in the GWAS implies the existence of an intermediate-frequency allele at the species level that causes multigenerational sterility at 25°C in laboratory conditions.

Naturally associated bacteria or microsporidia can rescue the Mrt phenotype of *C. elegans* wild isolates

The Mrt phenotype is intrinsically deleterious since it leads to sterility of the lineage, raising the question of how such polymorphisms can be maintained in natural populations. Temperature fluctuations may in part explain the suppression (Frézal et al, 2018). However, the phenotype was not found to be correlated with the temperature at isolation (Fig 1D), and some isolates with a particularly strong Mrt phenotype in the laboratory, such as JU775, were isolated under warm conditions. We, therefore, asked whether other ecologically relevant environmental features could affect the Mrt phenotype.

C. elegans is generally cultured in the laboratory using *E. coli* OP50 as a food source, yet it encounters a variety of other microbes in the wild (Barrière & Félix, 2014; Dirksen et al, 2016; Samuel et al, 2016). In the course of *C. elegans* collections from natural samples, we had noticed that the Mrt phenotype commonly appeared after bleaching the culture, a treatment that kills the associated microbes. We thus tested the impact of naturally associated microbes on the phenotype.

To this end, we freshly isolated new *C. elegans* strains from wild sources together with their microbial associates. We assayed the Mrt phenotype of the strains with their associated microbial fauna, or on *E. coli* OP50 alone after a bleach treatment (Fig 3A). The freshly isolated strains JU3224 from New Zealand and JU4134 from Santeuil could be propagated for more than 20 generations at 25°C with their associated microbes. However, after bleaching a culture on *E. coli* OP50, these two strains exhibited an Mrt phenotype at 25°C (Fig 3B and C).

From the JU3224 culture, we isolated a *Lelliottia* strain (JUb276), and from the JU4134 culture several bacteria, including an Actinomyces (JUb277), a *Pseudomonas* (JUb279), and a *Flavobacterium* (JUb281). We performed a new assay after reassociating the *C. elegans* strain with either *E. coli* OP50 or the isolated microbes, in groups or singly. The reassociation of single isolated bacterial clones to the bleached wild isolate partially or fully rescued the Mrt phenotype (Fig 3B and C, and EV3). *Lelliottia* JUb276 from the New Zealand strain could rescue the Mrt phenotype of the bleached Santeuil strain (Fig 3C). This observation suggests a common mechanism of interaction between these bacteria and *C. elegans* that rescued the latter's sterility.

Microsporidia are obligate intracellular parasites related to fungi. The microsporidia *Nematocida parisii* and *N. ausubeli* are commonly found associated with *C. elegans*, colonizing its intestinal cells and being transmitted horizontally via defecation and ingestion of spores (Fig 4A) (Troemel et al, 2008; Zhang et al, 2016). The latter isolated in association with *N. ausubeli* (Zhang et al, 2016). We

assayed the effect of their infection on the Mrt phenotype of JU775 and JU2526; these two strains exhibited a strong Mrt phenotype when cultured on *E. coli* OP50 without microsporidia. We used a temperature of 23°C because these microsporidia kill *C. elegans* too rapidly at 25°C. For both strains, the infection by either species of microsporidia strongly delayed the onset of sterility at 23°C (Fig 4B). Thus, rather than solely acting as a detrimental parasite, microsporidia conferred a context-dependent beneficial effect on their host. In addition, it is worth noting that they interfered with a multigenerational phenotype while infecting the soma.

As we previously reported, starvation or passage through the dauer diapause is common in the wild for *C. elegans* (Barrière & Félix, 2005; Félix & Duveau, 2012). Such treatments were reported to affect multigenerational phenotypes (Simon et al, 2014; Jobson et al, 2015; Demoinet et al, 2017). We tested whether they could affect the Mrt phenotype of the wild isolate JU775, and did not detect a significant effect in our conditions (Fig EV4).

Altogether, the results using naturally associated bacteria and microsporidia highlight the central role of microbial interaction on the phenotype of the host and the effect of infection of somatic tissues on the germline, thus breaching the Weismann barrier.

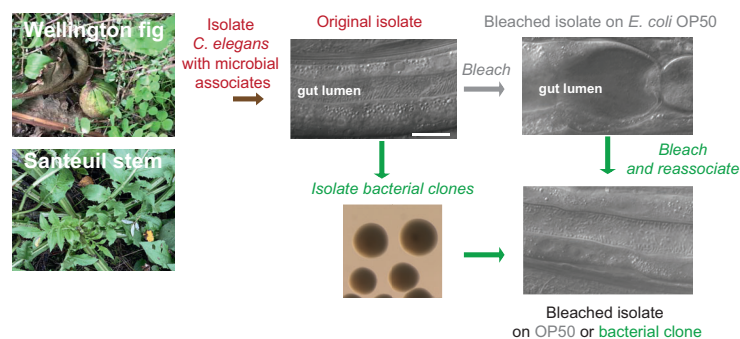
E. coli K-12 derivatives rescue the Mrt phenotype of *C. elegans* wild isolates compared to B lineage strains

Metabolic differences among *E. coli* strains of relevance to *C. elegans* nutrition are well documented (Yen & Curran, 2016; Zhang et al, 2017; Neve et al, 2020). We tested whether a different laboratory strain of *E. coli* could rescue the Mrt phenotype compared to the standard *E. coli* OP50 strain, a B-type strain (Brooks et al, 2009). Indeed, the K-12 strain MG1655 rescued the Mrt phenotype. The rescue was quantitative and depended on the strength of the Mrt phenotype. For the *C. elegans* JU4134 strain with an intermediate Mrt-value (around 8–10 generations on OP50 at 25°C), the rescue by *E. coli* MG1655 or the naturally associated bacteria JUb276 and JUb277 was nearly complete (> 20 generations for most replicates) (Fig 5A). For the JU775 strain with a strong Mrt phenotype (mean of four generations before sterility), the rescue at 25°C was significant but partial (Fig 5A). This partial rescue was likely the consequence of its stronger phenotype and not of a different interaction with the bacterium: the rescue of JU775 was indeed complete at 22°C, a temperature at which the severity of the Mrt phenotype on OP50 was similar to that of the JU4134 strain at 25°C (Fig 5B). Similarly, the Mrt phenotype of the MY10 strain used in Frézal et al (2018) was rescued at 22°C (Fig 5C).

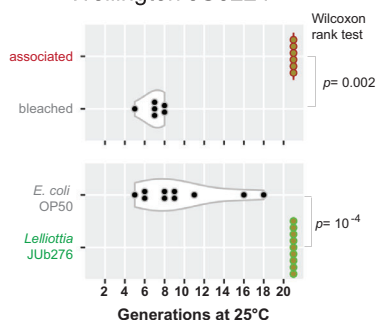
We focused on JU775 at 25°C because it allowed for shorter assays, and tested other *E. coli* strains. Culture on the *E. coli* B-type strain BL21(DE3) resulted in a strong Mrt phenotype, while the *E. coli* K-12 derivatives HT115, C600, CS180, and BW25113 partially rescued the phenotype (Figs 5D and E, and EV5). Mixing a K-12 and a B strain resulted in an intermediate Mrt phenotype (Fig 6A). These results indicated that the Mrt phenotype of wild isolates was revealed when they were cultured on an *E. coli* B-type strain.

E. coli B and K-12 strains differ in many aspects that are known to affect *C. elegans* phenotypes (Daegelen et al, 2009; Jeong et al, 2009; Studier et al, 2009; Maier et al, 2010; Reinke et al, 2010; Pinsky et al, 2011; Yoon et al, 2012; Yen & Curran, 2016; Heestand et al, 2018; Neve et al, 2020; Stühr & Curran, 2020;

A Isolation of naturally associated bacteria and reintroduction



B Wellington JU3224



C Santeuil JU4134

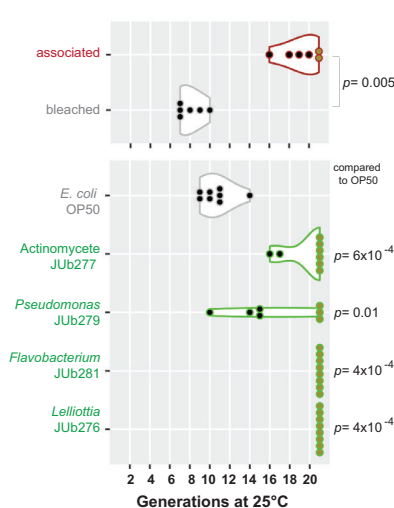


Figure 3. Suppression of the Mrt phenotype of newly isolated *C. elegans* by their associated bacteria.

A Schematic representation of the experiment, with color coding as in panels (B–C). *C. elegans* was collected from rotting fruit (JU3224) or stem (JU4134) and bacterial clones isolated from the culture. This experiment was performed independently with samples from two independent locations. Fig picture courtesy of Nathalie Pujol. Nomarski pictures show the presence of associated bacteria in the intestinal lumen of the nematode in the original culture and on the isolated bacteria (here JU3224 on JUb276), but none or few when cultured with *E. coli* OP50. Bar 10 μm .

B Mrt phenotype of the *C. elegans* isolate JU3224 in the presence of its original microbial associates (brown) on *E. coli* OP50 (gray) or reassociated with its own bacteria JUb276 (green).

C Mrt phenotype of the *C. elegans* Santeuil isolate JU4134 in the presence of its original microbial associates (brown) on *E. coli* OP50 (gray) or reassociated with isolated bacteria from its original culture or with JUb276 (green).

Data information: The Mrt phenotype is expressed in number of generations to sterility. Dots represent biological replicates, all assayed in the same experiment if in the same gray rectangle graph. The experiments of the bottom graphs of panels (B) and (C) were in addition performed in parallel. The suppression in JU4134 by JUb276 and JUb277 was replicated in an independent experiment shown in Fig 5A. The dark gold color of the dots highlights replicates that remained fertile. The *P*-values are for two-sided Wilcoxon rank tests compared to “bleached,” without adjustment for multiple testing.

Source data are available online for this figure.

Zhao *et al*, 2022). Especially, K-12 strains have longer lipopolysaccharides (LPS) in their cell wall than the B strains and the longer LPS of *E. coli* K-12 strains trigger p38/PMK-1 immune activation in *C. elegans* (Pradel *et al*, 1992; Aballay *et al*, 2003; Zhang *et al*, 2006; Maier *et al*, 2010; Leung *et al*, 2020; Ma *et al*, 2020). Using *E. coli* K-12 mutants affecting LPS synthesis, we did not detect an effect on the Mrt phenotype compared to the reference K-12 background (Fig EV5). Similarly, mutants that are defective in the curli fibers at the cell surface (Zhou *et al*, 2012; Chen *et al*, 2016) did not affect

the Mrt phenotype of *C. elegans* (see source data for Fig 5B, performed in parallel).

We explored the possibility that the Mrt phenotype was affected by a metabolite, the availability of which for *C. elegans* differed in the context of the different *E. coli* strains. As an obvious candidate, *C. elegans* fed with *E. coli* OP50 are known to display a lower vitamin B12 content, resulting in a variety of phenotypes that are rescued by vitamin B12 supplementation (Watson *et al*, 2014; Revtovich *et al*, 2019; Amin *et al*, 2020; Wei & Ruvkun, 2020; Nair

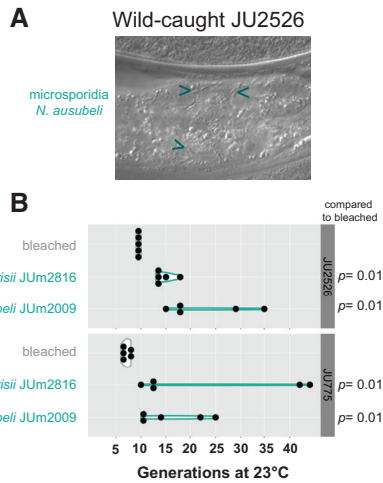


Figure 4. Infection by microsporidia suppresses the Mrt phenotype of *C. elegans* wild isolates.

A The *C. elegans* isolate JU2526 was originally associated with microsporidia *Nematocida ausubeli* (Zhang et al, 2016). Arrows indicate microsporidian spores in intestinal cells. Nomarski optics. Bar: 10 μ m.

B The Mrt phenotype of the *C. elegans* isolates JU2526 and JU775 is suppressed by infection with two *Nematocida* strains, at 23°C. The P -values are for two-sided Wilcoxon rank tests without adjustment for multiple testing compared to the bleached strain without microsporidia. The Mrt phenotype is expressed in number of generations to sterility. Dots represent biological replicates, all assayed in the same experiment if in the same gray rectangle graph. The two experiments are independent.

Source data are available online for this figure.

et al, 2022). Vitamin B12 supplementation did not suppress the Mrt phenotype of JU775 and JU4134 (Fig EV5).

Mutants in nuclear small RNA pathway or SET-domain genes are rescued by naturally associated bacteria and *E. coli* K-12

We further asked whether the rescue was specific to the Mrt phenotype of wild isolates or also concerned mutants in the N2 reference background with a temperature-sensitive Mrt phenotype.

We tested mutants in two different molecular processes: the *nrde-2* mutant affects the nuclear small RNA response involved in multigenerational silencing inheritance (Burton et al, 2011), while the *set-2* mutation affects a histone H3K4 methyltransferase (Robert et al, 2014; Caron et al, 2022). Both were rescued by the natural associate JUb276 and *E. coli* MG1655 (Fig 6A and B). For *set-2*, the results are consistent with Robert et al (2020), who found a suppression by *E. coli* HT115.

We previously showed that the strong Mrt phenotype of the MY10 wild isolate was partially explained by a deletion in the *set-24* gene (Frézal et al, 2018). We tested a *set-24* mutant in the N2 background (courtesy of Giulia Furlan and Eric Miska) and found that its Mrt phenotype was also rescued by the natural bacteria JUb276 and *E. coli* K-12 (MG1655) (Fig 6C).

Thus, we conclude that the Mrt phenotype of laboratory mutants in small RNA inheritance or SET-domain protein genes is suppressed by culture on bacteria other than *E. coli* OP50.

Multigenerational memory of the bacterial environment

In order to test whether the animals had a memory of their past bacterial environment, we shifted animals of the *C. elegans* JU775 strain cultured for several generations at 25°C on an *E. coli* K-12 strain (on which their Mrt phenotype was suppressed) to *E. coli* OP50 (Fig 7A). We performed this experiment twice, changing the *E. coli* strain (MG1655 or HT115) and the transfer conditions (with or without bleaching at the time of transfer). In both experiments (Fig 7A and B), the lines that were propagated for 10–20 generations at 25°C on an *E. coli* K-12 strain showed a rescued phenotype when transferred back to OP50. This indicates a multigenerational memory of the bacterial environment.

Discussion

Continuous distribution of the mortal germline phenotype

Expressed as the number of generations to sterility, the Mrt phenotype is a quantitative trait with a continuous distribution (Fig 1). Importantly, the laboratory reference strain N2 is not fully exempt from the phenomenon as in our experiments, it displayed the sterility phenotype in some replicates (Figs 2 and EV1).

The variance of the Mrt-value depends on its mean value (Fig 1), with a particularly high variance at intermediate Mrt-values. This makes conditions with intermediate Mrt-values particularly difficult to work with. Mrt phenotypes of intermediate strength are intrinsically variable because of the stochastic nature of the trait and its environmental sensitivity. In addition, the phenotype is sensitive to environmental conditions of past generations at 25°C (Fig 7), which further complicates the assays. Despite this experimental caveat, at least some variation in Mrt phenotype among isolates is of genetic origin and can be studied by linkage mapping using laboratory crosses (Frézal et al, 2018) or association in natural populations (here).

A major GWAS locus underlies this conditionally deleterious variation

The association study detected one major locus on chromosome III. We narrowed down the possible interval to 4.66–6.49 Mb, the association being stronger with the left part (4.66–5.4 Mb). The intermediate frequency of the candidate alleles derives from the GWAS approach, which cannot detect rare alleles, such as *set-24*, that are present in a single strain of the dataset.

The interval is in a region of low recombination in the center of *C. elegans* chromosomes (Rockman & Kruglyak, 2009; Noble et al, 2017), explaining a high linkage disequilibrium and the need for further recombination. SNPs were used as markers, and the candidate causal polymorphism(s) include structural variants (Cook et al, 2017). We note that the genome of wild strains with the “JU775” and “JU1793” chromosome III haplotypes, JU2526 and JU2600 (see Fig 5), were assembled from long-read sequencing data by Lee et al (2021), which can serve as tools for searching for candidate variants. The GWAS interval also contains a number of candidate single-nucleotide polymorphisms (Dataset EV1), including

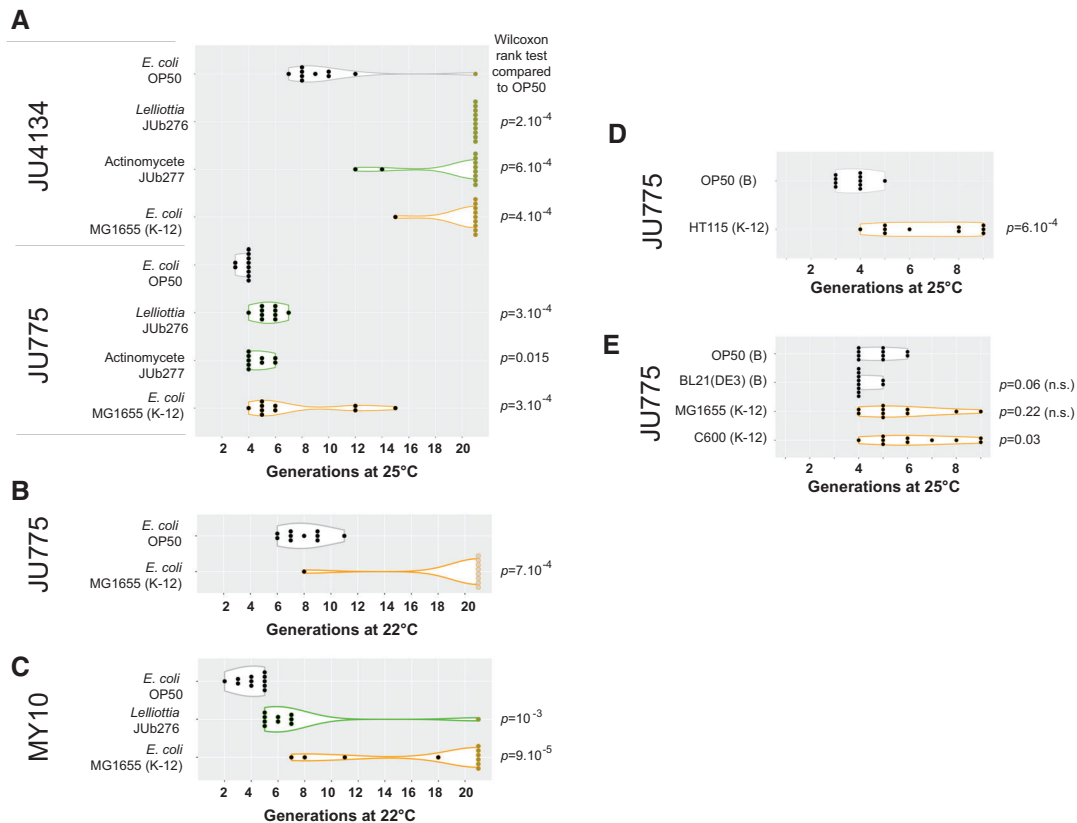


Figure 5. Suppression of mild and strong Mrt phenotypes by naturally associated bacteria and by *E. coli* K-12 strains.

A Effect of culture on bacteria JUb276, JUb277 (natural associates, green), and *E. coli* MG1655 strain (orange) on the Mrt phenotype of the *C. elegans* strains JU4134 and JU775 at 25°C compared to culture on *E. coli* OP50.

B Effect of *E. coli* MG1655 strain on the Mrt phenotype of the *C. elegans* strain JU775 at 22°C.

C Effect of *Lelliottia* JUb276 and *E. coli* MG1655 strain on the Mrt phenotype of the *C. elegans* strain MY10 at 22°C.

D Effect of culture on *E. coli* HT115 strain on the Mrt phenotype of the *C. elegans* strain JU775 at 25°C compared to culture on *E. coli* OP50.

E Effect of culture on *E. coli* K-12 lineage strains (orange) and a second B lineage strain (gray) on the Mrt phenotype of *C. elegans* JU775 at 25°C. The effect of *E. coli* MG1655 on JU775 is a replicate experiment of that in panel (A). The parallel assay of *E. coli* OP50 and MG1655 was repeated a third time and is shown in Fig 6A ($P = 8.10^{-5}$) and a fourth time with the same result ($P = 0.004$, $n = 10$ per treatment). Another *E. coli* K-12 background is shown in Fig EV5.

Data information: In all panels, the Mrt phenotype is expressed in number of generations to sterility. Dots represent biological replicates, assayed in the same experiment if in the same graph. The dark gold color of the dots highlights replicates that remained fertile. The P -values are for two-sided Wilcoxon rank tests without adjustment for multiple testing, compared to *E. coli* OP50. "n.s." = non-significant ($P > 0.05$).

Source data are available online for this figure.

in germline-expressed genes in which null mutations are known to cause an Mrt phenotype at 25°C: for example, statistically associated polymorphisms include non-synonymous substitutions in *morc-1*, *nhl-2*, and *set-2* (Herbette et al, 2017; Weiser et al, 2017; Davis et al, 2018; Robert et al, 2020). We caution that methods associated with CRISPR/Cas9 genome editing seemed to produce undesired effects on the Mrt phenotype, at least some of which disappeared after prolonged culture and/or freeze-thawing. Further work will be needed to identify the causal polymorphism(s).

Germline immortality in natural environmental conditions

In addition to the temperature dependence (Frézal et al, 2018), we detected here a strong biotic environmental component to the Mrt phenotype. In particular, the bacterial strain used to feed the nematode had a strong effect. The use of *E. coli* OP50 for *C. elegans*

culture dates from the "domestication" of *C. elegans* by Sydney Brenner (Brenner, 1974). Many *C. elegans* phenotypes have been recently described to depend on the bacterial culture environment, including the type of *E. coli* strain (Yen & Curran, 2016; Zhang et al, 2017; Neve et al, 2020). *E. coli* K-12 and B strains differ in a variety of features, several of which are known to affect other *C. elegans* phenotypes (Daegelen et al, 2009; Jeong et al, 2009; Studier et al, 2009; Reinke et al, 2010; Pinske et al, 2011; Yoon et al, 2012; Yen & Curran, 2016; Neve et al, 2020; Stuhr & Curran, 2020; Zhao et al, 2022). The use of *E. coli* screens (e.g., Virk et al, 2016; Han et al, 2017b; Qi & Han, 2018; Govindan et al, 2019; Warnhoff & Ruvkun, 2019; Zhang et al, 2019; Shin et al, 2020) may enable future studies to understand which environmental parameters explain variation in the Mrt phenotype. Importantly, this bacterial influence on the Mrt phenotype suggests that the relevant natural genetic variation may concern its metabolism, immunity, or

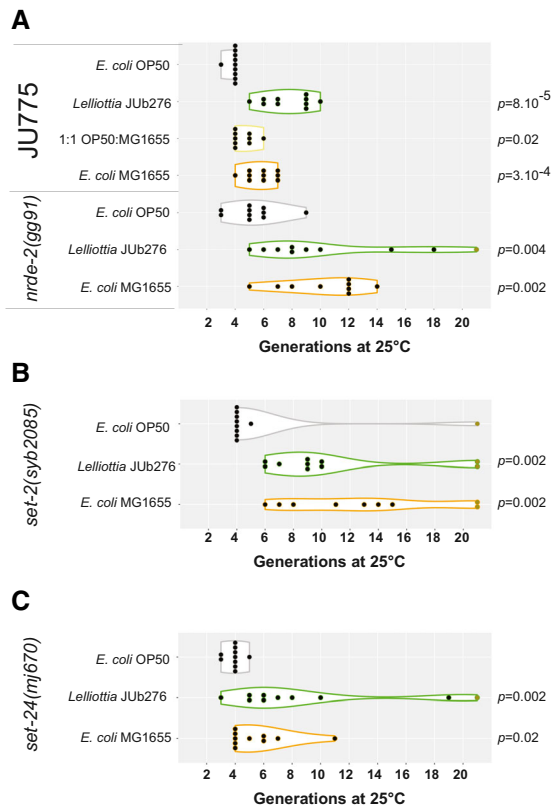


Figure 6. Suppression of the Mrt phenotype of *C. elegans nrde-2*, *set-2*, and *set-24* mutants by a naturally associated bacterium and *E. coli* K-12.

- A *nrde-2(gg91)* mutant in parallel with JU775. The phenotype of JU775 appears intermediate on a 1:1 mixture of the two *E. coli* strains compared to their pure culture.
- B *set-2(syb2085)* mutant.
- C *set-24(mj670)* mutant.

Data information: The Mrt phenotype is expressed in number of generations to sterility. Dots represent biological replicates, assayed in the same experiment if in the same graph. The dark gold color of the dots highlights replicates that remained fertile. The *P*-values are for two-sided Wilcoxon rank tests without adjustment for multiple testing, compared to *E. coli* OP50. These experiments were performed a single time.

Source data are available online for this figure.

sensory system, in addition or in combination with gene expression regulation by small RNAs and histone modifications. Variation in metabolism and interaction with microbes possibly link to small RNA and histone modification pools (Tauffenberger & Parker, 2014; Han et al, 2017a; Kaletsky et al, 2020; Nono et al, 2020; Legue et al, 2022; Wan et al, 2022).

A particular case is that of obligate intracellular intestinal parasites. *N. parisii* and *N. ausubeli* microsporidia are strong suppressors of the Mrt phenotype and hence have a conditional positive effect on population growth. These horizontally transmitted microsporidia are frequently encountered with *C. elegans* (Zhang et al, 2016). They infect the intestine and not the germline, thus showing an effect of the soma on germline maintenance. This constitutes one more example of an absence of a soma-to-germline “Weismann” barrier in *C. elegans* (e.g., Rechavi et al, 2014; Devanapally et al, 2015; Jobson et al, 2015; Moore et al, 2019; Gabaldon et al, 2020).

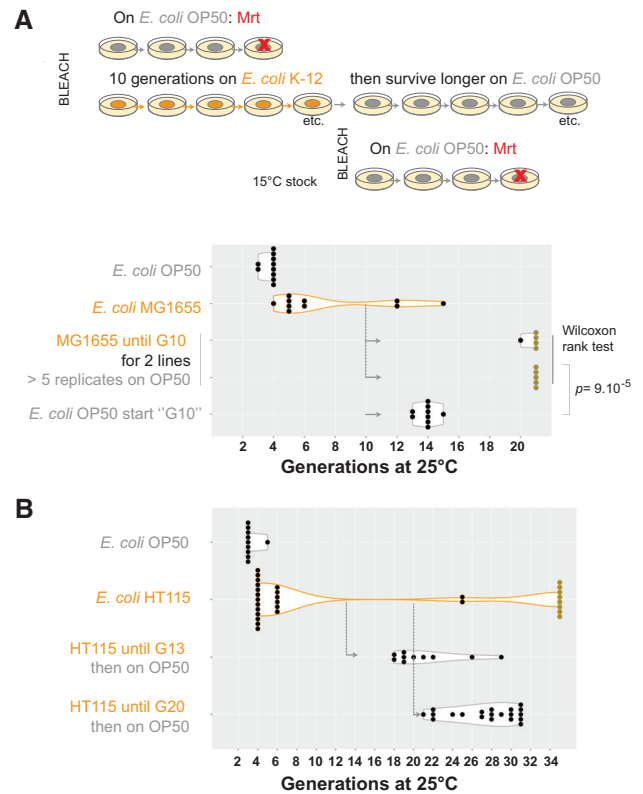


Figure 7. Multigenerational memory of the bacterial environment.

- A The Mrt phenotype of JU775 was assayed on *E. coli* OP50 or an *E. coli* K-12 strain (MG1655) at 25°C. After 10 (A panel) generations on the MG1655 strain, 5 replicates of the two surviving lines were maintained on the MG1655 strain or transferred back to OP50 (gray arrows) using a bleach treatment. The experiment is the continuation of that in Fig 5A. Control from a 15°C culture maintained without starvation (“15°C stock”) was bleached in parallel (labeled “*E. coli* OP50 start ‘G10’” in the graph of panel A). Two-sided Wilcoxon rank test comparing the transferred versus control replicates: *** for $P < 0.001$.
- B The Mrt phenotype of JU775 was assayed on *E. coli* OP50 or an *E. coli* K-12 strain (HT115 carrying irrelevant RNA interference plasmids, as indicated in the Source data file) at 25°C. After 13 or 20 generations on the HT115 strain, replicates of surviving lines were maintained on the HT115 strain or transferred back to OP50 (gray arrows) without bleach treatment. A single replicate and two replicates were assayed from G13 and G20, respectively.

Data information: The Mrt phenotype is expressed in number of generations to sterility. Each dot represents a biological replicate. The dark gold color of the dots highlights replicates that remained fertile. Panels (A) and (B) are independent experiments using slightly different conditions.

Source data are available online for this figure.

The surprising maintenance of a conditional deleterious phenotype

Being strongly deleterious, the Mrt phenotype is likely shielded in the wild, explaining how associated polymorphisms can be maintained at intermediate frequency.

The ts-Mrt phenotype can therefore be considered as a consequence of the standard *C. elegans* laboratory culture conditions—useful as a tool to reveal laboratory mutations and natural polymorphisms in small RNA inheritance and histone modification pathways

(e.g., *set-24*), as well as to study germline maintenance requirements. Specific environmental conditions may also reveal it in the wild.

Mutation accumulation lines can be used to measure the ease with which a given phenotype can be altered by *de novo* mutation and, by comparison with natural populations, allow inference of natural selection. With this aim in mind, Saber *et al* (2022) used spontaneous mutation accumulation lines in *C. elegans* starting from two wild-derived strains with a non-Mrt phenotype. They found a single mutation line with a strong Mrt phenotype, likely caused by a frameshifting insertion in *nrde-2*, and none with a weak Mrt phenotype. Since many wild isolates have at least a weak Mrt phenotype, they concluded that the Mrt phenotype may be underbalancing selection in the wild. Balancing selection is consistent with a varying environmental context of selection of the underlying genetic variation.

The ability to transmit non-genetic information allows the species to transiently provide environmental cues to a variable number of generations. Variation in small RNA inheritance and chromatin (as with *set-24*) may be selected differentially by specific temporal sequences of environmental variation. The co-occurrence of relevant chromosome III haplotypes on multiple continents and in local populations (Table EV2) is suggestive of balancing selection; however, a linked locus other than that causing the Mrt phenotype may be involved. Beyond the large diversity of Argonaute genes and RNAi mechanisms (Winston *et al*, 2000; Tijsterman *et al*, 2002; Yigit *et al*, 2006; Félix *et al*, 2011; Paaby *et al*, 2015; preprint: Chou *et al*, 2023), these nematodes may have evolved variation modulating the memory of non-genetic information.

Multigenerational sterility

We previously examined cellular features of germline degeneration along the mortal germline process of *C. elegans* wild isolates: germline reduction, defects in chromosome pairing, and excess of unresolved double-stranded breaks (Frézal *et al*, 2018). These cellular features resemble the Mrt phenotype of mutants in small RNA inheritance and histone-modifying enzymes, which involves abnormal regulation of gene expression, including repetitive elements (McMurchy *et al*, 2017; Wallis *et al*, 2019), as well as genome stability (Herbette *et al*, 2017) and germline pluripotency (Robert *et al*, 2014). Their Mrt phenotype appears, however, distinct from that of the *prg-1/piwi* mutant (for which the cause of sterility is debated (Barberan-Soler *et al*, 2014; McMurchy *et al*, 2017; Heestand *et al*, 2018; Barucci *et al*, 2020; Reed *et al*, 2020; Spichal *et al*, 2021; Wahba *et al*, 2021): especially, the latter does not show temperature dependence and is suppressed by starvation (Simon *et al*, 2014).

The temperature-dependent Mrt phenotype itself is a manifestation of memory of the temperature environment across generations. Whether this temperature dependence reflects a variation in metabolism, germ granules, chromatin compaction, nucleic acid hybridization, or any other process remains to be investigated.

In addition to the cumulative nature of the Mrt phenotype over generations, we demonstrated that a wild *C. elegans* isolate keeps a further multigenerational memory of the bacteria on which the strain was cultured at 25°C. This suggests that its Mrt phenotype is not only due to a metabolic requirement during the

tested generations but may result from metabolic carryover of the physiological or epigenetic state of the animals. Such memory has been observed in *C. elegans* using various experimental paradigms (Rechavi *et al*, 2014; Jobson *et al*, 2015; Ma *et al*, 2019; Moore *et al*, 2019; Baugh & Day, 2020; Gabaldon *et al*, 2020; Zhang *et al*, 2021). Further work will be necessary to study its molecular basis. A possible mechanism involves changes in the pools of small RNAs and histone modifications (Dodson & Kennedy, 2019), or their metabolic precursors. Alternatively, other processes, such as the quality or quantity of mitochondria (Zhang *et al*, 2021), may be at play in this multigenerational environmental memory.

Materials and Methods

C. elegans culture and strains

C. elegans was cultured on 55 mm NGM agar plates (Stier-nagle, 2006) seeded with 100 µl of a saturated culture of *E. coli* OP50 grown overnight in LB at 37°C, except if otherwise indicated. *C. elegans* wild isolates for the association analysis were obtained from CGC, Michael Ailion, Erik Andersen, Cori Bargmann, Christian Braendle, Elie Dolgin, Asher Cutter, Matt Rockman, Paul Sternberg, or our own collection. The *set-2(syb2085)* mutant (Caron *et al*, 2022) was a gift of Francesca Palladino, and the *set-24(mj670)* mutant of Giulia Furlan and Eric Miska. The *nrde-2(gg91)* mutant (Burton *et al*, 2011) was obtained from CGC.

Bleaching was performed by spotting gravid adult hermaphrodites or embryos onto an NGM plate seeded with *E. coli* OP50 and immediately adding 50 µl of the bleach/NaOH solution (Stiernagle, 2006).

E. coli strains

OP50 was acquired from Paul Sternberg's lab in 1994. MG1655 was received from Javier Apfeld's lab, C600 from John Chapman's lab, and BL21(DE3) from Bertrand Ducos (ENS). HT115 was acquired from Andy Fire and transformed with the L4440 vector carrying various inserts (Timmons *et al*, 2001).

Mortal germline assays

Mortal germline (Mrt) assays were carried out as in Frézal *et al* (2018), transferring three L4-stage hermaphrodites at each generation onto a new plate. Before the start of the assay, the strains were maintained at 15°C with food for two or more generations prior to shifting at the higher temperature. The assays were performed at 25°C unless otherwise indicated. With this method, animals are never starved. We recorded the generation *n* of culture at 25°C at which no larva was produced (Generation *n* or *G_n*). The assays were generally stopped once the fertility of the 20th generation was scored (reported as G21 in the graphs). The assays were carried out without blinding; however, the animals were transferred as larvae before sterility can be observed. The developmental stage was controlled at the time of allocation to treatment, either by bleaching or by transfer of individuals. Contaminated replicate lines were removed.

Genome-wide association analysis

We used data from several Mrt assays performed in different laboratories across several years (see Fig EV1). Each Mrt assay was conducted in a number of replicates, from 1 to 6. Each assay represents a block, indicated by the date, experimenter, and the laboratory where the assay was performed. In total, we studied 12 blocks, performed by four experimenters: Marie-Anne Félix (MAF), Lise Frézal (LF), Emilie Demoinet (ED, data from Frézal et al, 2018), and Sayran Saber (SS, data from Saber et al, 2022). The experiments were conducted in four different laboratories: ours in Paris, Eric Miska's lab in England, Christian Braendle's in Nice, and Charles Baer's lab in Florida.

To plot the distribution of the Mrt-value across the dataset (Fig 1B), the mean Mrt-value for each strain was calculated as the mean of all the Mrt-values of any experiment where the strain was assayed.

For the GWAS, the strains that were fertile after 20 generations were considered non-Mrt and we set their Mrt-value at 30 generations. We removed the outlier Bergerac strain derivative RW7000 that was sterile at the first generation due to a temperature-sensitive *zyg-12* mutation that is unrelated to the Mutator phenotype of some of its derivatives (Fatt & Dougherty, 1963; Mori et al, 1988; Malone et al, 2003; Nigon & Félix, 2017; Frézal et al, 2018).

We used the *lme4* package (Bates et al, 2015) in R to fit generalized linear mixed-effect models using a Poisson distribution in Rstudio version 1.2.5001 (R Core Team, 2015). Block, which is confounded with experimenter, was significantly associated with phenotype (deviance = 3,304.0 against 3,451.7 for the reduced model with isotype as the sole random effect). For GWAS, we extracted isotype BLUPs (best linear unbiased predictions) using the *ranef* function on the full model: `glmer(value ~ (1|block) + (1|isotype), Mrt_ALL, family = "poisson")`. Residuals were approximately normal (Shapiro–Wilk $W = 0.988$).

To perform the GWAS, we used the SNPs from the 2020-08-15 version of the hard-filtered isotype vcf at CeNDR (Cook et al, 2017), which we filtered using the PLINK toolset (<https://zzz.bwh.harvard.edu/plink/>). The 132 strains represented 119 different isotypes according to CeNDR. We took all SNPs with a minimum allele frequency of 0.05 and removed markers in complete linkage disequilibrium with the *indep* pairwise command using a sliding window size of 100,000 bp, a count of 50,000 variant per window, and a maximum r^2 of 0.99. We performed further GWAS on specific regions on chromosome III (from 4 to 10 Mb), using the SNPs from the imputed variants vcf from the 2020-08-15 version of CeNDR.

We used the GridLMM package to test SNP associations with the Mrt BLUPs (Runcie, 2018), controlling for population structure with an additive kinship matrix. The significance threshold was calculated from 1,000 phenotype permutations (using the *permut*s function). The Kendall correlation test between the mean Mrt-values of the strains and their mean temperature was calculated with the values from Table EV1.

We used the *snpEff* and *SnpSift* tools to annotate and filter the effect of each SNPs given by the GWAS fine mapping (Cingolani et al, 2012), with the database of the Wormbase WS263 version from CeNDR as a reference genome (Davis et al, 2022).

Haplotype analysis

We extracted SNVs in the region of 4.5–6 Mb on chromosome III among our strains from the soft-filtered imputed isotype VCF file (CeNDR 2022/02/16 release) (Cook et al, 2017) and filtered out variants that were below the 5% minor allele frequency using *BCFtools* (v.1.9) (Li, 2011) and *PLINK* (v1.9) (Purcell et al, 2007; Chang et al, 2015). The regional haplotype of each strain was inferred using *IBDseq* (v.r1206) (Browning & Browning, 2013). The haplotype network was built using *SplitsTree4* (Huson & Bryant, 2006).

Introgressions

All crosses were performed at 18°C. To produce the introgression lines, chromosome III of JU775 was introgressed into the N2 background by a minimum of six rounds of backcrossing followed by marker genotyping on the different chromosomes (see primers and genotypes in Tables EV3 and EV4). Two independent introgressions were made, creating the strains JU3194 and JU3195.

The *mjIs31[pie-1::gfp::h2b]* transgene was initially introduced by others using an artificial *Mos1* transposon insertion site at 8.4 Mb on chromosome II of the N2 reference strain (Ashe et al, 2012), creating the strain SX461. For another project, we introduced this *mjIs31[pie-1::gfp::h2b]* transgene into the above-introgressed strain JU3195 via two independent crosses, creating the strains JU3394 and JU3395.

To use as a control, this transgene was backcrossed six times into the wild isolate JU775, creating the independent strains JU3306 and JU3307 (Table EV1). (Although *set-24* may be irrelevant in the case of JU775, we took care that these strains carry JU775 alleles in the region of this gene, which carries a partial deletion in MY10 and is located on the same chromosome as the *mjIs31[pie-1::gfp::h2b]* transgene.)

Generation of recombinants

Recombination within chromosome III was performed by crossing the males of the introgressed lines JU3395 (JU775 chrIII>SX461) to SX461 hermaphrodites. The crosses were performed at 20°C and the following generations were kept at 18°C to prevent selection of the non-Mrt alleles. For each cross, one F1 heterozygote hermaphrodite was selected randomly, and about 100 F2 individuals were singled and genotyped for a recombination event between two markers on chromosome III at 5.4 and 11.0 Mb (Table EV4). We then selected recombinant lines that were homozygous at both sites. To this end, we singled 8 F3 animals from the lines homozygous at one site but heterozygous at the other site; we genotyped these F3 animals and selected one with a homozygous recombinant genotype. Multiple recombinant lines were thus obtained: JU4312, JU4336-41, and JU4348.

Microbe isolation and rDNA sequencing

New *C. elegans* isolates and their associated microbes were collected from natural substrates as described in Félix & Duveau (2012) and Barrière & Félix (2014), derived from single hermaphrodites. JU3224-3228 were isolated from samples of rotting vegetal matter from New Zealand, 1–3 weeks after sample collection in April 2017.

JU4134 was isolated from the decaying stem of a plant of the Asteraceae family in Santeuil (France) on October 30, 2020. In this case, the sample was placed on an NGM plate with *E. coli* OP50 a few hours after collection. *C. elegans* is generally fully homozygous at all loci in natural populations (Barrière & Félix, 2005; Richaud et al, 2018); to make sure our lines were isogenic, strains were selfed by isolation of a single hermaphrodite for 4–5 generations before further use.

To assess the microbial contribution to the Mrt phenotype of JU4134, a procedure via a microbial preparation was used because the experimenter did not expect that single bacteria could rescue the Mrt phenotype and wished to go stepwise from the most ecologically relevant environment. A microbial preparation was first made by incubating the nematodes overnight with 10 mM tetramisole in 15 ml tubes, with mild mixing on a rotator. The nematode corpses were pelleted by centrifugation at 1000 g. The preparation was used for seeding NGM plates or for further isolation of bacteria. This tetramisole procedure did not kill all nematodes, so seeded plates were inspected after a few days and discarded if they contained a nematode that had survived.

Microbial strains JUb277–283 were then isolated from the JU4134 microbial preparation by streaking on 90 mm NGM plates without bacteria. Individual colonies were further isolated by streaking on a new plate. *Pseudomonas* JUb283 was isolated directly from the JU4134 culture and is a wrinkler morph compared to the non-wrinkler JUb279. Bacteria showing different colony and cell aspects when observed with the dissecting microscope or under Nomarski optics were retained. The bacteria were given a Jub identifier.

The bacterial strain JUb276 was isolated from the unbleached culture of JU3224 by streaking the microbial culture surrounding the nematodes onto 90 mm NGM plates. The plates were incubated at room temperature. The streaking procedure was repeated to isolate single colonies. One colony was then used as JUb276 and a culture derivative was frozen.

PCR primers for bacterial rDNA sequencing are in Table EV3. NCBI accession numbers are ON110486–90. Sequences of JUb277 and JUb282 were identical, as were those of JUb279, JUb280, and JUb283.

Bacteria were grown overnight in liquid LB with shaking, at 37°C for *E. coli*, and at 25°C for the other bacteria. A 100 µl drop of the culture was then spotted in the center of a 55 mm NGM nematode culture plate (50 µl in some experiments, as indicated in Source data). The plates were left at room temperature for 2–3 days and stored in a cold room before use. Care was taken to equilibrate the plates at the relevant temperature before use for the Mortal germline assay.

Mortal germline assays in the presence of microbes

Several methods were used to expose nematodes to microbes during the Mortal germline assays; to each corresponds a color code in the figures. The assay could be performed with the original microbial fauna (maroon) before the *C. elegans* culture was bleached. All other methods started with a bleached nematode culture. In the first case, the plates were seeded with the microbial tetramisole preparation (see above), in the absence of *E. coli*, and the nematode culture was bleached on this plate. In the second method (green), the plate was seeded by one or several microbial strains isolated as above,

and cultured overnight in LB at 25°C in a shaker. The latter method was also used for culture on different *E. coli* strains.

The *C. elegans* stock was maintained at 15°C on OP50 without starvation, and for two to three generations with the relevant bacteria at 15°C, before the mortal germline assay was started by shifting to 25°C (see details corresponding to each experiment in Source data).

Microsporidial spores were prepared and quantified as in Zhang et al (2016). Twenty uninfected young adults (i.e., prior to first egg formation) were transferred to a 55 mm NGM plate seeded with *E. coli* OP50. Five million microsporidial spores in 100 µl distilled water were placed onto the *E. coli* lawn. The cultures were then incubated at 15°C. The infection symptoms were checked by Nomarski optics 72 h after inoculation. Ten L4 larvae from the infected population were transferred to a fresh plate and moved to 23°C (Generation 1) and then 10 L4 animals were transferred onto fresh plates at each generation (every 2–3 days, generally 3 transfers per week). Uninfected individual L4 larvae were also tested in parallel. We used 23°C because the detrimental effects of the microsporidia were too high at 25°C. In this experiment, the transfer was stopped when 10 L4-stage animals were not available.

E. coli HT115 bacteria carrying (irrelevant) RNA interference plasmids, and OP50, were grown overnight in liquid LB with shaking, at 37°C, and then seeded (100 µl) onto NGM plates supplied with 1 µM IPTG. Before the start of the assay, the *C. elegans* strains were maintained at 15°C on OP50. To initiate the Mrt assays, five gravid hermaphrodites were spotted onto freshly prepared IPTG plates (24–32 h), immediately adding 50 µl of the bleach/NaOH solution (Stiernagle, 2006) before incubating plates at 25°C. The following steps are identical to the Mortal germline assays described above, transferring three L4-stage animals at each generation onto a new plate. In order to assess the memory of past bacterial environment, at G13 and G20, for all the fertile lineages, three L4-stage animals were transferred back to OP50-seeded plates at 25°C; the following steps are similar to the Mrt assay with the transfer of three L4-stage animals at each generation onto a new OP50-seeded plate.

Supplementation of chemicals

Two forms of vitamin B12, hydroxocobalamin hydrochloride (Sigma #95200) and methylcobalamin (Sigma M9756), were each dissolved at 5 mg/ml in ddH₂O and filter sterilized using a 0.22 µm filter. One hundred microliter of this stock solution was spread on the plate before seeding *E. coli* OP50, or added on top of the *E. coli* lawn, as indicated in Source data. The final concentration once the vitamin has diffused into the agar is 37 nM, above the 20 nM used in Giese et al (2020) and 12.8 nM in Wei & Ruvkun (2020). How each experiment was started can be found in Source data.

We tested the activity of these vitamin B12 forms using a reporter, especially since the plates were stored at 4°C until the end of the Mrt experiment. The *acdH-1::GFP* reporter of *C. elegans* strain VL749 fluoresces brightly on control plates at 25°C but was repressed on the plates with either vitamin B12 form, even when plates had been stored. To test whether the vitamin also entered animals with a JU775 background, the transgene was introduced in the JU775 background by 10 successive crosses, yielding strain JU4271. The GFP fluorescence was also repressed by both vitamin B12 forms on this strain.

Statistical analysis of Mrt assays

The Mrt trait quantified as the number of generations to sterility has a low variance when the number is small, a high variance when it is larger, and again a low variance when none of the replicates become sterile by 20 generations. We thus used a conservative rank test for statistical testing, specifically in R the `wilcox.test` with continuity correction. Given the difficulty of these multigenerational tests and the loss of some replicates due to contaminations, some of the experiments ($n < 5$) did not include sufficient power for statistical testing with this method. We only compared assays run in parallel, represented in a given graph, except for the initial GWAS analysis, where blocks were accounted for, as detailed above.

Data availability

The data generated in this study are in the Source Data files of each figure. Reagents generated during this study are available upon request to MAF (felix@bio.ens.psl.eu). The R code for the GWAS is available at https://github.com/MSglio/Mrt_GWAS_code. The rDNA bacterial sequences are listed in Table EV3 and available at NCBI with ID# ON110486-90:

<https://www.ncbi.nlm.nih.gov/search/all/?term=ON110486>;
<https://www.ncbi.nlm.nih.gov/search/all/?term=ON110487>;
<https://www.ncbi.nlm.nih.gov/search/all/?term=ON110488>;
<https://www.ncbi.nlm.nih.gov/search/all/?term=ON110489>;
<https://www.ncbi.nlm.nih.gov/search/all/?term=ON110490>.

Expanded View for this article is available [online](#).

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Author contributions

Lise Frézal: Conceptualization; investigation; writing – review and editing.

Marie Saglio: Data curation; formal analysis; validation; investigation; visualization; writing – original draft. **Gaotian Zhang:** Formal analysis; investigation; writing – review and editing. **Luke Noble:** Software. **Aurélien Richaud:** Investigation. **Marie-Anne Félix:** Conceptualization; data curation; formal analysis; supervision; funding acquisition; investigation; visualization; writing – original draft.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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Expanded View Figures

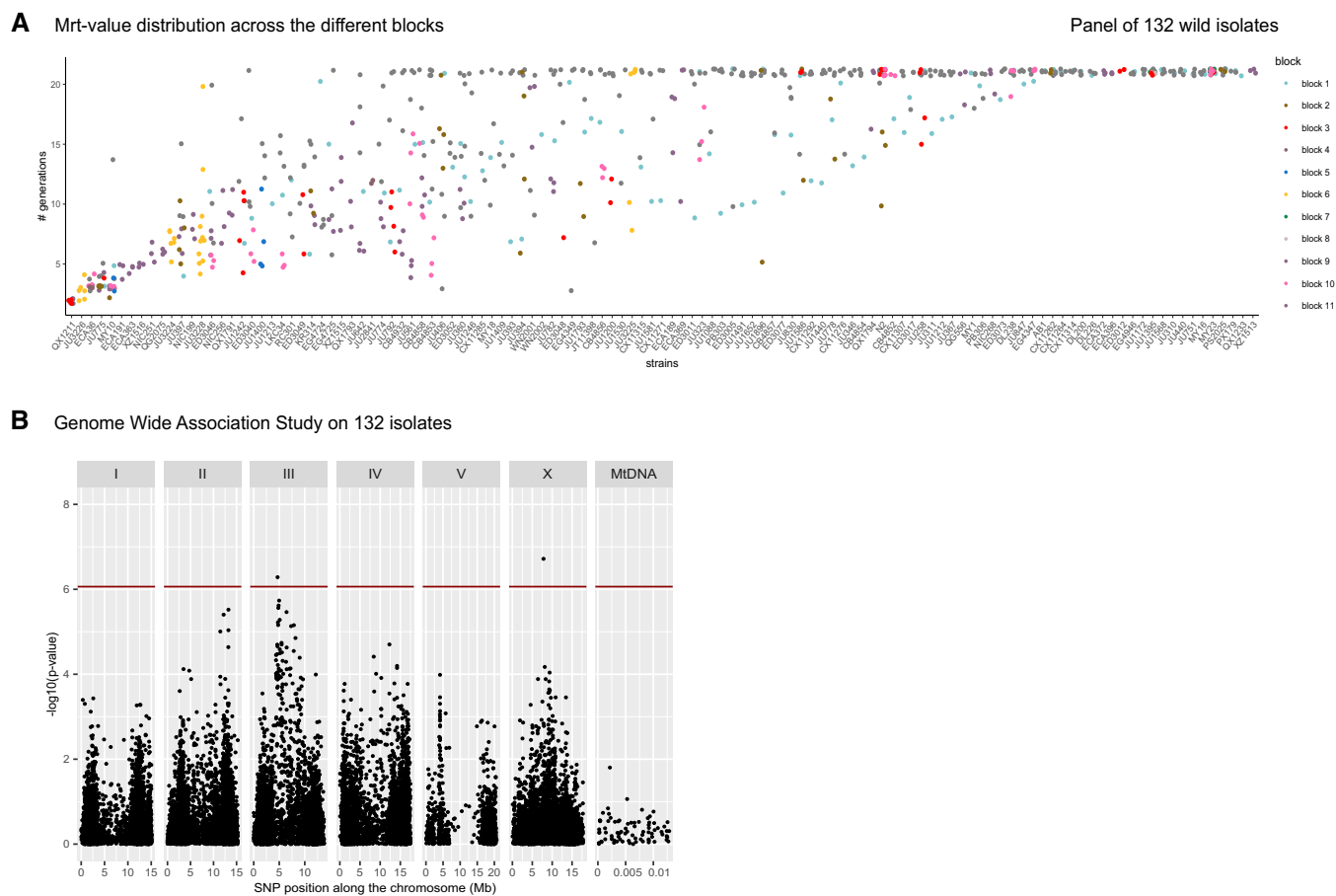


Figure EV1. Mrt-values of the full panel of 132 wild isolates, including the Pacific divergent strains.

- A Distribution of the Mrt-values of each experimental block for each strain. The Mrt phenotype is expressed in number of generations to sterility. Each dot represents a biological replicate. The 11 blocks correspond to experiments performed at different times and are represented by different colors.
- B Genome-wide association study on the full panel of 132 isolates. The plot represents the significance value of the association between the tested single-nucleotide polymorphisms (SNPs) plotted along the six chromosomes and mitochondrial DNA (MtDNA), using the generalized linear mixed model. The red line indicates the significance threshold at $P < 0.05$, estimated using permutations. The genomic position is expressed in megabases (Mb). The signal on chromosome X is driven by the divergent isolates.

Figure EV2. Haplotype structure of *C. elegans* wild isolates in the chromosome III genomic region.

- A Color coding of the haplotype structure along the chromosome III region for the strains used in GWAS analysis in Fig 1 ($n = 113$). The strains are homozygous due to selfing hence their genome is represented by a single haplotype. The vertical dashed line shows the position of the III:4677491 SNP. The left and right panels show strains with the reference (N2) or alternative allele at this SNP, respectively. Blank lines correspond to single haplotypes that were not called (e.g., if they do not share a haplotype with any other strain of the set).
- B Phenotypic distribution for each haplotype at the III:4677491 position (thus, merging haplotypes of panel A that differ elsewhere in the interval). Chromosome III haplotypes are named below the graph after one representative strain; the individual dots correspond to the mean Mrt phenotype of the different wild strains carrying this haplotype, expressed in number of generations to sterility ($n = 113$). The interquartile range is displayed as a box, the median as a horizontal line, and the whiskers represent $1.5 \times$ the interquartile range. Some strains with the "JU775" chromosome III haplotype do not show a strong Mrt phenotype, suggesting that other polymorphisms contribute to phenotypic variation.
- C Haplotype network of the same region showing one or few representative strains per haplotype, including the divergent Pacific strains (found at the bottom of the plot) ($n = 132$). Genotype data are from CeNDR (www.caendr.org). The strain name is color coded according to the allelic state of the III:4677491 SNP (orange, N2 reference; blue, alternative). Recombination within the region in the non-Pacific set is visible in the representations of the (A) and (C) panels.

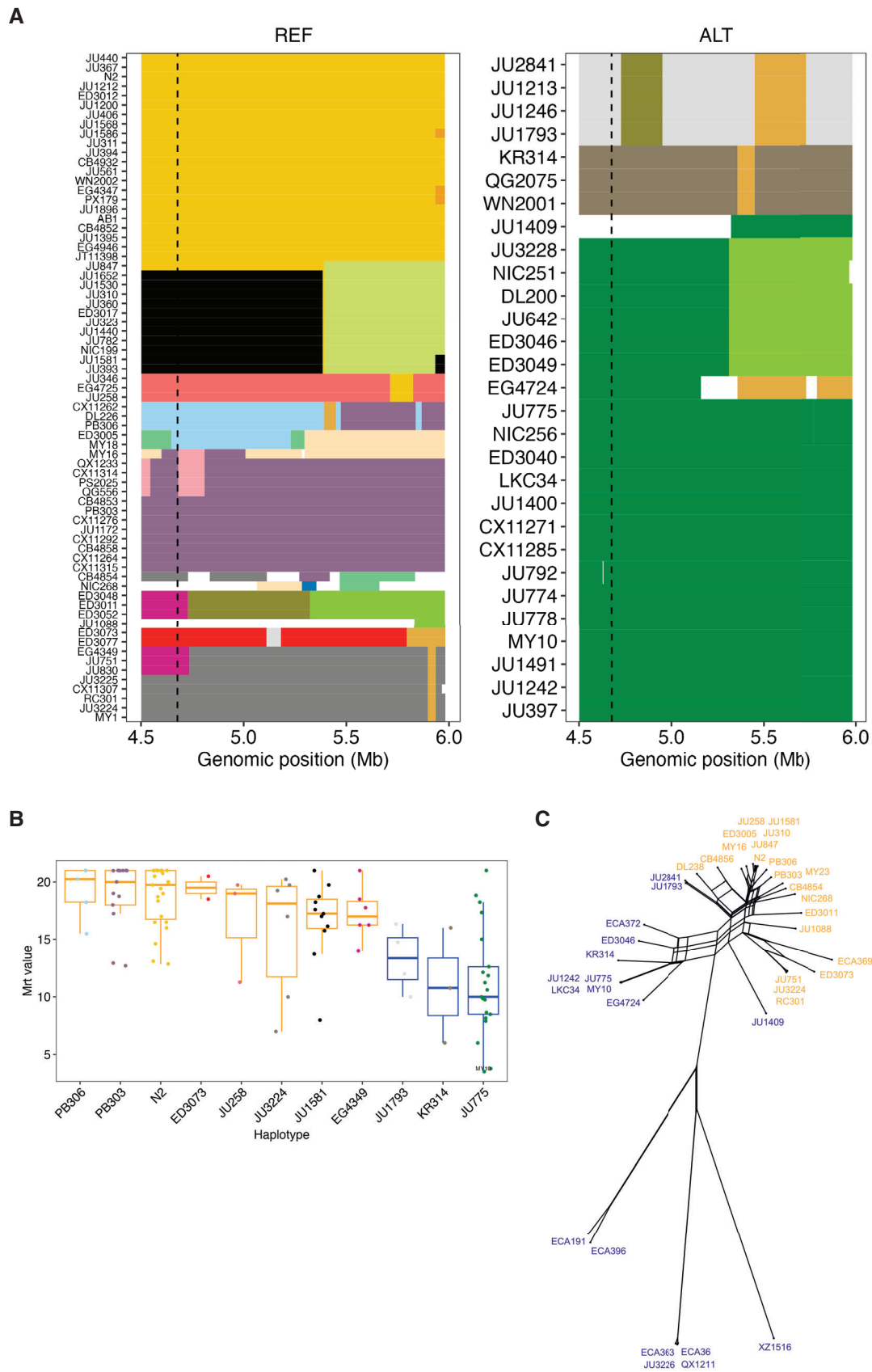


Figure EV2.

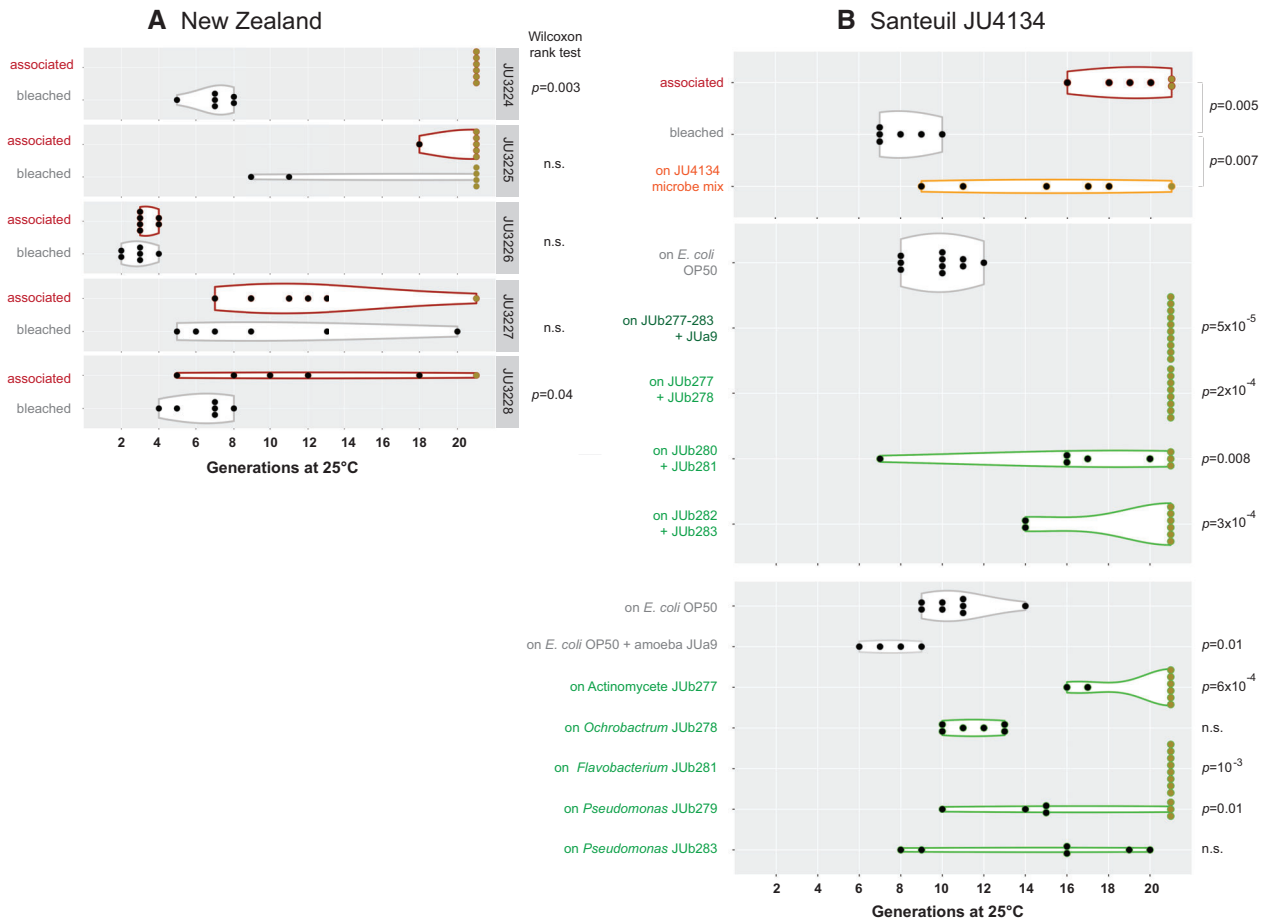


Figure EV3. Additional experiments during isolation of *C. elegans* wild isolates and their associated microbes.

- A The five *C. elegans* strains that were isolated from New Zealand were scored in parallel. JU3224 showed the clearest rescue of the Mrt phenotype by associated microbes, was used for isolation of a bacterial clone, and is shown in Fig 3B.
- B Intermediate steps in the isolation of bacteria from JU4134. The different graphs represent independent experiments. The top graph shows the effect of adding a levamisole-treated microbial preparation of the JU4134 culture; the middle graph shows the assay on a mix of isolated bacteria and eukaryote or pairs of bacteria; and the bottom graph shows the experiment on single clones of bacteria. The experiments in the top and bottom graphs are shown in part in Fig 3C.

Data information: The Mrt phenotype is expressed in number of generations to sterility. In all graphs, each dot represents a replicate. The dark gold color of the dots highlights replicates that remained fertile. The *P*-values of Wilcoxon rank tests without adjustment for multiple testing are shown. n.s.: non-significant ($P > 0.05$).

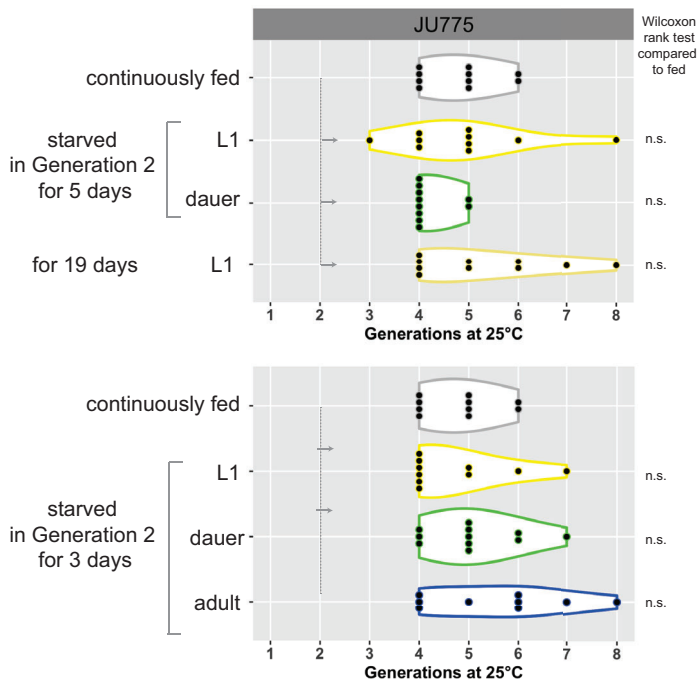


Figure EV4. Starvation does not strongly rescue the Mrt phenotype of the wild isolate JU775.

The two graphs represent two experiments performed successively. The “continuously fed” controls were started from a 15°C culture at different times. The first experiment was performed in parallel to that in Fig 5C. The second experiment serves as a control for the 19-day starvation treatment of the first experiment fed at the same time as the second experiment was started at 25°C. The Mrt phenotype is expressed in number of generations to sterility. Each dot represents a replicate. Wilcoxon rank tests without adjustment for multiple testing compared to non-starved controls were non-significant ($P > 0.05$). The pairing of fed and starved lines is indicated in Source data.

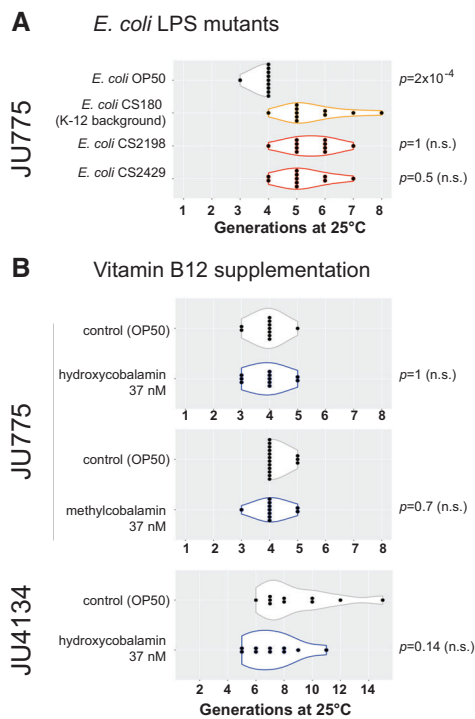


Figure EV5. Test of the effect of *E. coli* lipopolysaccharide (LPS) mutants and vitamin B12 supplementation on the Mrt phenotype of wild isolates.

- A** *E. coli* LPS mutants. The *E. coli* CS180 strain in the K-12 background suppresses partially the Mrt phenotype compared to the OP50 strain. The two LPS mutant strains in the CS160 background, CS2198 and CS2429, still suppress the Mrt phenotype. *P*-values of Wilcoxon rank tests without adjustment for multiple testing compared to the *E. coli* CS160 treatment are shown.
- B** Vitamin B12 supplementation does not suppress the strong Mrt phenotype of JU775 or the weaker phenotype of JU4134. The panel shows three independent experiments. The *P*-values of Wilcoxon rank test compared to the control are shown.

Data information: For all panels, the Mrt phenotype is expressed in number of generations to sterility. Each dot represents a biological replicate. The experiment in panel (A) was performed a single time. Panel (B) shows three independent experiments.