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Systematic comparison of bacterial feeding strains for increased yield of *Caenorhabditis elegans* males by RNA interference-induced non-disjunction

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ABSTRACT

Rare *Caenorhabditis elegans* males arise when sex chromosome non-disjunction occurs during meiosis in self-fertilizing hermaphrodites. Non-disjunction is a relatively rare event, and males are typically observed at a frequency of less than one in five hundred wild-type animals. Males are required for genetic crosses and phenotypic analysis, yet current methods to generate large numbers of males can be cumbersome. Here, we identify RNAi reagents (dsRNA-expressing bacteria) with improved effectiveness for eliciting males. Specifically, we used RNAi to systematically reduce the expression of over two hundred genes with meiotic chromosome segregation functions, and we identified a set of RNAi reagents that robustly and reproducibly elicited male progeny.

1. Introduction

Caenorhabditis elegans is a simple, multi-cellular animal commonly used in a variety of scientific investigations. This genetically tractable organism is particularly useful for studies related to meiotic chromosome segregation, as the number of X chromosomes is used to establish gender in this organism. Male progeny (5AA; XO) naturally arise as a result of X chromosome non-disjunction during meiosis in the hermaphrodite parent (5AA; XX). Additionally, the male frequency in a strain can reflect its multi-generational history of exposure to mutagens or temperature and the duration of its propagation as a self-fertilizing hermaphrodite stock [1–3]. In the laboratory, non-disjoined males are observed at a frequency of less than one in five hundred wild-type animals [4,5]. Because a self-fertilizing hermaphrodite typically produces only three hundred progeny, many individuals will sire no male offspring. The rarity and elusiveness of males can thus be problematic for those biochemical, phenotypic or genetic studies that require males in abundance.

Several methods have been devised to increase the yield of males in wild-type and mutant strains; however, each method has limitations [6]. Mating strategies, for instance, can increase male numbers: in a mating of hermaphrodites with males, half of the resulting offspring can be males. Similarly, *him* mutations (High Incidence of Males) can be crossed into a strain of interest. Because *him* genes are required for proper chromosome disjunction, strains with *him* mutations produce male offspring more frequently [4,5]. While mating strategies provide an effective means to elicit more males, these strategies can prove onerous and time-consuming. This is especially the case for strains that harbor transgenes in addition to mutations in multiple genes. When crosses are performed between such strains and wild-type or *him* males, animals that are homozygous for all loci must be re-isolated in subsequent generations in order to establish a male-producing stock. A second means of increasing male numbers involves altering the environmental conditions. More male progeny can be observed when animals are exposed to elevated temperatures or ethanol [7,8], which can be advantageous for direct propagation of homozygous male progeny from strains with complex genotypes. Such treatment can increase the yield of males to 2–5% of

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the population; however, results can vary [9]. Furthermore, many mutants display sensitivity to these environmental conditions. A third means of increasing male numbers uses RNAi to elicit non-disjunction. For example, when animals ingest dsRNA corresponding in sequence to *him-14*, a gene required for crossover of homologous chromosomes, male progeny are observed [10]. Ingestion of *him-14* dsRNA-expressing bacteria (*him-14* "food") for multiple generations conveniently allows for the direct production of males (as many as 5–7%) from complex strains. Because RNAi is reversible, studies in subsequent generations should not be compromised by *him-14* deficiency.

him-14 food is not the only RNAi reagent that can be used to induce non-disjunction. In an RNAi-based screen using bacteria expressing dsRNA corresponding to genes on chromosome IV, our laboratory previously observed Him phenotypes for several bacterial clones. Since then, our lab has successfully utilized one of these bacterial clones, *klp-15* food, to isolate fertile males from a number of different strains [11]. In our hands, *klp-15* food was more effective in generating males than *him-14* food. We hypothesized that other bacterial feeding strains might be more effective than *klp-15*, and further improvements in the "male food" methodology might be possible by systematically assessing dsRNA-expressing bacterial clones that can induce non-disjunction.

2. Materials and methods

2.1. Strains used in these studies

NL3531 [*rde-2(pk1657)*], PD8186 [*rde-2(ne221)*], NL1820 [*mut-7(pk720)*], and NL917 [*mut-7(pk204)*]. EG4322 [*ttTi5605* II; *unc-119(ed3)* III]. Wild type strains used: XX935 [N2 received from *C. elegans* Genetics Center Feb. 2006. DR subclone of CB original, Tc1 pattern I].

2.2. RNAi delivery using bacterial "feeding"

Plasmids used as templates for dsRNA production were derived from the Source BioScience LifeSciences *C. elegans* RNAi library [12]. The targeted genes are associated with Him phenotypes in mutant or RNAi-treated animals. The phenotypic information was obtained from publications and data sets deposited online using WormMart and WormMine data mining tools and literature search features in Wormbase: (releases WS232-240) and the literature search tools of PubMed: www.ncbi.nlm.nih.gov [13,14], including the RNAi database: http://aquila.bio.nyu.edu/cgi-bin/rnaidb/index.cgi [15]. Insert sizes were verified by PCR. CelRNAi (http://biocompute.bmi.ac.cn/CelRNAi/) was also consulted to validate the clones [16]. Bacterial colonies from fresh transformations of plasmids into HT115(DE3) cells were used for each feeding experiment. dsRNA expression was induced in liquid culture as described, and the induced culture was placed onto standard NGM plates supplemented with tetracycline, ampicillin, and Isopropyl-β-D-1-thiogalactopyranoside (IPTG) [17–19]. Wild-type worms were placed as L1/L2 larvae on the bacterial lawn (4 worms per plate), and the F1 progeny were counted and scored for males. The animals were reared at 22 °C on feeding plates. In general, experiments performed at 25 °C resulted in higher percentages of males, mostly as a result of increased lethality of progeny due to non-disjunction of autosomes. Exposure of animals to dsRNA for multiple generations by transferring treated animals to a freshly prepared plate of dsRNA-expressing bacteria also resulted in sterility of many treated animals, an increase in numbers of dead embryos, and higher percentages of males. Thirty-nine bacterial strains produced no males in three independent experiments, with each experiment consisting of three to four plates.

2.3. Assessing effectiveness of "male food"

The bacterial feeding method is inherently inconsistent. We routinely utilized two protocols known to improve the effectiveness of RNAi-by-feeding and improve experimental consistency: we used recently transformed HT115(DE3) host bacterial cells (from transformation plates that have been stored for less than one week), and we used fresh preparations of feeding plates (used one day after seeding). In our experiments, the presence of males may not directly reflect the effectiveness of the knock-down for several reasons. Our RNAi phenocopy of interest (Him) is an indirect readout for reduction of gene expression for genes involved in chromosome disjunction, and non-disjunction events are, for the most part, unpredictable, if not random. A Him phenocopy requires non-disjunction of an X-chromosome, and non-disjunction of autosomes leads to sterility of treated animals and lethality in disjoined progeny-factors that can influence the number of viable male progeny obtained. For most of the genes targeted in this study, RNAi by feeding resulted in reduced brood sizes (0–100 offspring observed from three parental worms) in at least one experiment. For this reason, we do not report observations of male progeny as a percentage of the brood size, as this is a better indicator of autosomal aneuploidy, and not necessarily X-chromosome disjunction and male production. For example, while we observed high percentages of males from treated animals with drastically reduced brood sizes, the total number of males produced in such experiments was not increased over the number obtained from animals with larger brood sizes. (1 male in 7 progeny was observed for *ula-1* knockdown.) Our goal was to identify feeding strains that consistently produced the greatest number of males, not necessarily the highest percentage of males. Because the feeding protocol in general, and production of a Him phenocopy in particular, has inherent experimental inconsistencies, within and between experiments, we compared median and average results to help identify reliable male-producing bacterial food.

2.4. Simultaneous delivery of two dsRNAs ("supermale food")

Three plasmids (derivatives of plasmid L4440 [18]) were generated with *C. elegans* DNA sequences flanked by bacteriophage T7 promoters. pLT651 harbors *klp-16/him-8* sequences; pLT652 *dhc-3/him-8* , and pLT653 has *dhc-3/klp-16*. The gene sequences were obtained from the Source BioScience LifeSciences *C. elegans* RNAi library [12], and include 1169 bp from exons 2–4 of *klp-16*, 1019 bp from exons 3–6 of *him-8*, and 1180 bp from exons 24–28 of *dhc-3*. The plasmids were transformed into HT115(DE3) bacteria, dsRNA expression was induced [18,19], and the number of male progeny were tabulated as described above. Because these RNAi reagents were the most consistent in generating males, we refer to them as "supermale food".

3. Results and discussion

To identify prospective "male foods", we first compiled a list of genes for which Him phenotypes have been observed in the corresponding *C. elegans* mutants or in animals treated with gene-specific dsRNA. Approximately one hundred fifty *C. elegans* genes were identified in this way (Table S1). RNAi tools are available for most of these genes in the form of dsRNA-expressing plasmids contained in bacterial strains from the Source BioScience LifeSciences *C. elegans* RNAi library [12] (Table S1). Some of the plasmid clones harbor overlapping *C. elegans* gene sequences and likely lead to simultaneous knock-down of multiple genes, while others may contribute to the silencing of genes within a homologous family (Tables S1 and S2). The potential for multiplex RNAi in these

instances increased the predicted number of gene targets in our studies to more than two hundred (highlighted in Table S1). Multiplex RNAi may result in a weaker than anticipated Him response, as unintended targets may have no functional relevance to chromosome disjunction mechanisms.

The relative effectiveness of the prospective male foods was ascertained by preparing plates with dsRNA-expressing bacteria as described [17–19], allowing wild-type *C. elegans* to ingest the induced bacteria, and tabulating the number of male progeny (Table 1 and Table S3). While the Him phenocopies produced in our experiments were reproducible, the experimental deviations highlight the intrinsically variable nature of RNAi by feeding (Table 2). A number of different factors may contribute to experimental inconsistency: batch-to-batch variability in the accumulation of dsRNA within the bacterial strains, animal-to-animal differences in the amount of dsRNA ingested or the amount that is made bioavailable, or RNAi response differences in individual animals due to unanticipated background mutations in genes with RNAi-related functions [20–23]. For the particular case of producing Him phenocopies, the presence of males may not directly reflect the effectiveness of the knockdown. For example, effective reduction in the expression of genes that function as part of redundant disjunction mechanisms might lead to unpredictable, if not random, occurrences of non-disjunction. By contrast, for genes with essential chromosome disjunction functions affecting autosomes, variability in RNAi response is advantageous: a weaker RNAi response would allow for the isolation of males, while a stronger RNAi response would be expected to induce sterility. Indeed, reductions in brood size or sterility were observed in at least one plate per experiment for most strains. For example, in a *ula-1* feeding experiment, fewer than ten progeny (including one male) was observed on each of two plates. Because of the variability in brood sizes in the RNAi knockdown experiments, we do not report the Him phenotype as a percentage of males in the progeny, which is a better indicator of the extent of aneuploidy and not necessarily a capacity to generate large numbers of males, as most disjunction mechanisms affect autosomes as well as the X-chromosome. Indeed, we did not observe large numbers of males in experiments with the smallest brood sizes. Instead, we report the overall number of males observed as well as the median, as an indicator of effective and reproducible male production (Tables 1, 2 and S3). By our reckoning, *klp-15* food, and the related sequence *klp-16*, ranked highest in ability to promote non-disjunction. Other kinesin motor protein family members were similarly effective (Table 1).

We next reasoned that the methodology might be further improved by simultaneous targeting of two genes from this dataset. We constructed plasmids with opposable T7 promoters flanking a two-gene hybrid sequence of *klp-16* and *him-8*; *dhc-3* and *him-8*; or *klp-16* and *dhc-3* (Table 2). We selected pairwise combinations of genes that likely function in different or opposing chromosome disjunction pathways in order to avoid genetic

Table 2
Simultaneous knockdown of two genes leads to more consistent male production. Three plasmids (derivatives of plasmid L4440 (Timmons and Fire 1998)) were generated with *C. elegans* DNA sequences flanked by bacteriophage T7 promoters. pLT651 harbors *klp-16/him-8*; pLT652 *dhc-3/him-8* , and pLT653 has *dhc-3/klp-16*. dsRNA expression was induced (Timmons et al. 2001; Timmons and Fire 1998) and the number of male progeny were tabulated as in Table 1. These RNAi reagents were consistent in generating males, and we refer to them as "supermale food".

Target Gene	Temp	Average #Males (Experiment)	Average #Males (Per plate)	Median #Males (Experiment)
him-8/klp-16	25 °C	46 (SD = 7)	14 (SD = 9)	45 (n = 3)
him-8/klp-16	22 °C	4 (SD = 0.6)	1 (SD = 1)	4 (n = 3)
him-8/klp-16	20 °C	3.7 (SD = 1.8)	1 (SD = 3)	0 (n = 3)
him-8/dhc-3	25 °C	8 (SD = 1.4)	2.5 (SD = 1)	9 (n = 3)
him-8/dhc-3	22 °C	0.3 (SD = 1.2)	0 (SD = 0.3)	0 (n = 3)
him-8/dhc-3	20 °C	0 (SD = 0)	0 (SD = 0)	0 (n = 3)
klp-16/dhc-3	25 °C	19 (SD = 13.5)	17 (SD = 13.5)	81 (n = 3)
klp-16/dhc-3	22 °C	**5** (SD = 5.5)	6 (SD = 4.5)	29 (n = 3)
klp-16/dhc-3	20 °C	1.5 (SD = 2)	2 (SD = 2.1)	7 (n = 3)

Table 1
The most effective "male foods". The median and average number of males per experiment was determined in order to assess the effectiveness of each dsRNA-expressing bacterial strain, as the bacterial feeding method is inherently inconsistent. The observational units included an experiment consisting of three to four plate replicates; assessments were made for individual plates as well. The Him phenocopy can be enhanced by maintaining animals on "male food" for multiple generations, however, in some instances, this leads to increased Sterility. The doubly marked strains indicate those reagents with high rankings for male production as assessed by the experimental median, the average per experiment, and average per plate. Strains indicated by darker coloration indicate those with high rankings as assessed by the experimental median and average per plate. Strains indicated in bold ranked highly in average male production per experiment and per plate.

Target Gene	Off-target Sequences	Median #Males (Experiment)	Average #Males (Experiment)
klp-16	*klp-15*	10 (n = 4)	**19.8** (SD = 27)
klp-15	*klp-16*	5 (n = 5)	**15.4** (SD = 22)
klp-7		0 (n = 5)	**7.0** (SD = 18)
zim-1	*zim-2, zim-3*	6.5 (n = 2)	**6.5** (SD = 3.5)
him-4		2 (n = 6)	**5.2** (SD = 6.6)
htp-1	*htp-2*	0 (n = 4)	**4.8** (SD = 9.5)
syp-2		2 (n = 4)	**4.5** (SD = 6.5)
syp-1		2 (n = 3)	**4.0** (SD = 5.3)
rad-50		1 (n = 5)	**4.0** (SD = 7.9)
rec-8		1 (n = 6)	**3.8** (SD = 7.0)
ZK328.4		0 (n = 5)	**3.8** (SD = 8.0)
him-17		1 (n = 5)	**3.6** (SD = 4.0)
hcp-2		2 (n = 4)	**3.5** (SD = 4.7)
him-19	*Y95B8A.6*	0.5 (n = 4)	**3.3** (SD = 5.9)
dhc-3		1 (n = 5)	**3.2** (SD = 3.5)
him-8		5 (n = 5)	**3.0** (SD = 9.6)
coh-1		0 (n = 6)	**3.0** (SD = 6.4)
klp-18	*klp-10*	1 (n = 5)	**2.8** (SD = 3.0)

Table 3
Infertility in *mut-7* and *rde-2* Him males is not observed in males obtained using "male food". *mut-7* and *rde-2* males were readily obtained due to the inherent Him phenotype of the mutants. *mut-7* and *rde-2* males were also obtained using *klp-16*"male food". (As *mut-7* and *rde-2* mutants are not completely RNAi defective, males can be isolated using bacterial feeding.) Each cross plate contained one hermaphrodite and seven males; male progeny, as well as non-Uncs (where appropriate), were indicative of a successful mating. These results, which reflect more extensive observations, implicate a link between mutant-induced defects that promote non-disjunction in early germline development and movement defects that affect mating ability later in adult males.

Strain	Source of males	Hermaphrodite	Successful matings
rde-2(pk1657)	Mutation-induced males	Wild type	0/6
rde-2(ne221)	Mutation-induced males	Wild type	0/3
rde-2(pk1657)	Mutation-induced males	*unc-119(ed3)*	7/7
rde-2(ne221)	Mutation-induced males	*unc-119(ed3)*	3/3
rde-2(pk1657)	RNAi-induced males	Wild type	10/10
rde-2(ne221)	RNAi-induced males	Wild type	9/10
mut-7(pk720)	Mutation-induced males	Wild type	0/4
mut-7(pk204)	Mutation-induced males	Wild type	0/2
mut-7(pk720)	Mutation-induced males	*unc-119(ed3)*	0/5
mut-7(pk204)	Mutation-induced males	*unc-119(ed3)*	2/2
mut-7(pk720)	RNAi-induced males	Wild type	8/10
mut-7(pk204)	RNAi-induced males	Wild type	9/10

enhancements that would lead to sterility due to non-disjunction of autosomes. Each of these "supermale foods" was consistently effective in eliciting male progeny when the experiments were performed at 25 °C (8–20% males) (Table 2). The *klp-16/dhc-3* food was the most effective food (2–5% of the progeny were males), even at lower temperatures (20 °C). All males from the three "supermale" RNAi tools were fertile.

A particularly successful application of the RNAi-based strategy to produce males led to productive matings using *mut-7* and *rde-2* mutant males [11]. Even though *mut-7* and *rde-2* mutants naturally display Him phenotypes, the mutant males do not procreate. By contrast, those *mut-7* and *rde-2* males that are derived from "male food" are fertile. These contrasting phenotypes in the two mutant male populations (males produced by RNAi-induced non-disjunction versus *mut-7/rde-2*-induced non-disjunction) might be considered epigenetic in nature, as the two male populations have the same genotype, yet different male fertility phenotypes. Thus, in addition to the defects in *mut-7* and *rde-2* mutants that contribute to germline non-disjunction, the animals also harbor defects that persist into later developmental stages, affecting fertility in adult males. Indeed, endogenous siRNAi defects in *mut-7* have been correlated with odor adaptation in AWC neurons of adults [24]. We investigated the nature of the fertility defects in *mut-7* and *rde-2* mutant males, and we found that the males were capable of producing progeny when the hermaphrodite parent was *unc* (paralyzed), suggesting that mutant males are defective in movements that are required for effective mating (Table 3). Nonetheless, an ability to generate males using RNAi affords an ability to perform productive matings using homozygous *mut-7* or *rde-2* males and non-Unc hermaphrodites.

4. Conclusion

Here, we have compiled a comprehensive list of genes that are required for proper chromosome disjunction in the *C. elegans* germ line. From this list, we have identified better tools for the facile and direct production of fertile males. This list should serve as a good starting point for the production of large numbers of males from different strains. However, lab-to-lab as well as strain-to-strain variability should be anticipated, and investigators should realize the potential of background mutations, including those that affect RNAi [20–23]. The number of effective "male foods" identified improves flexibility in experimental design; for example, selective use of certain "male foods" may help avoid interactions between the RNAi phenocopy and mutations in the strain of interest, as well as improve male fertility, as we demonstrated for *mut-7* and *rde-2* males.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.07.023.

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