Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*

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Abstract

Genetic interference mediated by double-stranded RNA (RNAi) has been a valuable tool in the analysis of gene function in *Caenorhabditis elegans*. Here we report an efficient induction of RNAi using bacteria to deliver double-stranded RNA. This method makes use of bacteria that are deficient in RNaseIII, an enzyme that normally degrades a majority of dsRNAs in the bacterial cell. Bacteria deficient for RNaseIII were engineered to produce high quantities of specific dsRNA segments. When fed to *C. elegans*, such engineered bacteria were found to produce populations of RNAi-affected animals with phenotypes that were comparable in expressivity to the corresponding loss-of-function mutants. We found the method to be most effective in inducing RNAi for non-neuronal tissue of late larval and adult hermaphrodites, with decreased effectiveness in the nervous system, in early larval stages, and in males. Bacteria-induced RNAi phenotypes could be maintained over the course of several generations with continuous feeding, allowing for convenient assessments of the biological consequences of specific genetic interference and of continuous exposure to dsRNAs. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

A variety of species exhibit a defense response in which a double stranded RNA (dsRNA) 'trigger' produces a premature loss of endogenous RNAs with extended regions of sequence identity to the trigger (see Fire, 1999 for a review). A striking feature of dsRNA-triggered genetic interference processes (RNAi) in *C. elegans* is the ability of the interference to 'spread' to cells that are some distance from the initial site of dsRNA delivery. The first indication of this spreading effect came from the observation that dsRNA injected into the body cavity produced interference phenotypes throughout the injected animal (Fire et al., 1998). A later and more dramatic demonstration of a spreading effect was observed after feeding animals food that contained dsRNA (Timmons and Fire, 1998) or after soaking animals in dsRNA (Tabara et al., 1998).

The manner in which an organism responds to nucleic acid encountered in food is of interest from biological and technical perspectives. The predominant component of the *C. elegans* diet in the laboratory is bacteria; therefore, introduction of dsRNA into the *C. elegans* diet has involved engineering bacteria to produce dsRNA (Timmons and Fire, 1998). Although specific interference for several genes was evident in earlier studies with dsRNA-producing bacteria (Timmons and Fire, 1998), the observed phenotypes were limited in penetrance and expressivity. While demonstrating that ingested dsRNA is competent to trigger interference, these initial experiments left unresolved the question of whether loss-of-function phenotypes could be effectively produced by ingested dsRNA. To address this question, we sought to increase the effectiveness of the bacterial feeding technique by several means, including modifications that increase the concentration of dsRNA in the bacterial cell. We have found that a bacterial strain lacking the dsRNA-specific endonuclease RNaseIII can be cultured to produce high levels of specific dsRNA and that these bacteria can effectively trigger strong and gene-specific phenotypic responses when fed to *C. elegans*.

2. Materials and methods

2.1. C. elegans strains

All *C. elegans* strains were derived from the wild-type N2 strain of Brenner (Brenner, 1974). Three transgenic *gfp* strains were used in experiments for which interference with *gfp* was tested: PD4251 *(ccIs4251)* is homozygous for a *myo-3::gfp* transgene insertion (Fire et al., 1998); VH41 is homozygous for an *unc-119::gfp* transgene insertion *(rhIs13)* (a gift from Dr H. Hutter, Max Planck); and PD8047 harbors an extrachromosomal array with a *let-858::gfp* reporter *(ccEx8047)* (Kelly et al., 1997). *him* strains used to produce males were: *him-1(e879) I, him-3(e1256) IV, him-5(e1467) V, him-5(e1490) V, him-6(e1423) IV, him-8(ay29) IV, him-10(e1511) III,* and *him-14(it44) II* (Hodgkin et al., 1979; Kemphues et al., 1988).

2.2. Bacterial strains

Bacterial strains used were:

BL21: *ompT hsdS$_B$* (r$_B^-$m$_B^-$) *gal dcm* (Studier et al., 1990; Tabor and Richardson, 1985; Wood, 1966).

W3110: IN(*rrn*D-*rrn*E)1 (Dasgupta et al., 1998; Takiff et al., 1989).

SDF204: W3110, *rnc$^+$ TD1-17*::ΔTn*10*(Dasgupta et al., 1998; Takiff et al., 1989).

HT115: W3110, *rnc14*::ΔTn*10*(Dasgupta et al., 1998; Takiff et al., 1989).

DH5αF′: φ80d*lacZ*Δm15Δ(*lacZ*YA-argF)U169 *rec*A1 *end*A1 *hsd*R17(r$_K^-$m$_K^+$) *deo*R *sup*E44 *thi*-1 *gyr*A96 *rel*A1{F′} (Life Technologies).

HB101: *mcr*B *mrr hsd*S20 (r$_B^-$m$_B^-$) *leu*B6 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*-5 *mtl*-1 *rec*A14) (Boyer and Roulland-Dussoix, 1969).

TG1: *sup*E *thi*-1 Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM)5 (r$_K^-$m$_K^-$) {F′ *tra*D36 *pro*AB$^+$ *lac*I^{q}ZΔM15} (Stratagene).

SCS110: rpsL (Str′) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) {F′ traD36 proAB lacI^{q}ZΔM15} (Stratagene).

XL1-Blue MRF′: Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* {F′ *pro*AB *lac*I^{q}ZΔM15 Tn*10* (Tet^r)}^{d} (Stratagene).

Tn10 confers tetracycline resistance to the SDF204, HT115, and XL1-Blue MRF′ strains. Strains were modified to express T7 RNA polymerase from an IPTG-inducible promoter by site-specific integration into the chromosomal *attB* site of a λDE3 derivative carrying an IPTG-inducible copy of the phage gene 1 encoding T7 polymerase (Studier and Moffatt, 1986).

2.3. Plasmids

Plasmids were constructed using standard cloning techniques. Intermediate cloning steps were performed in DH5α′. GFP and *gfp* abbreviations refer to the protein and gene sequences, respectively, of the green fluorescent protein from *Aequorea victoria*.

The **L4440 (pPD129.36)** cloning vector contains two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site and was made using standard cloning techniques (Timmons and Fire, 1998).

L4417 (pPD128.110) has 0.7 kb of GFP coding sequence inserted between two convergent T7 promoters.

L4431 (pPD129.17) contains two copies of the 0.8 kb GFP coding region in tail-to-tail inverted orientation, separated by 0.8 kb of *unc-22* gene. A T7 promoter and T7 transcriptional terminator flank the *gfp* inverted repeat.

pLT61 contains 0.8 kb of *unc-22* inserted into the L4440 vector.

pLT76 contains two copies of a 0.6 kb *unc-22* sequence in tail-to-tail orientation separated by 0.9 kb of *gfp* and vector sequences. A T7 promoter and a T7 transcriptional terminator flank the *unc-22* inverted repeat.

L4366 (pPD128.18) contains 0.8 kb of *unc-22* and a short linker (from *gfp*) flanked by T3 and T7 promoters.

L4400 (pPD128.117) contains two copies of a 0.6 kb *unc-22* sequence in head-to-head orientation separated by 0.8 kb of *gfp* and vector sequences. A T7 promoter and a T3 promoter flank the *unc-22* inverted repeat.

pLT62 contains 0.8 kb of the *unc-54* gene inserted into the L4440 vector.

pLT63 contains 0.8 kb of the *fem-1* gene inserted into the L4440 vector.

pLT64 contains 1.2 kb of the *hlh-1* cDNA inserted into the L4440 vector.

Plasmids were transformed into bacterial hosts using standard CaCl$_2$ transformation protocols and plated on antibiotic-containing LB-agar plates. All these plasmids contained the β-lactamase gene. 75 μg/ml of ampicillin was included in plates and in liquid media. For XL1-Blue MRF′, SDF204, and HT115 strains, tetracycline was used at 12.5 μg/ml in both media and in plates.

2.4. dsRNA feeding conditions

A 2 ml 2 × YT culture containing appropriate antibiotics (see above) was inoculated with a single colony of host cell + plasmid and cultured overnight at 37°C. The culture was diluted more than 100 fold and allowed to grow to $OD_{600} = 0.4$. Isopropyl-β-ᴅ-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the culture was incubated with shaking for 2–4 h at 37°C. After supplementing with additional ampicillin, tetracycline, and IPTG, the cells were either directly applied onto agar plates or were concentrated by centrifugation and then applied. Unless otherwise noted, plates were composed of standard NGM/agar media (Brenner, 1974) supplemented with 50–100 μg/ml ampicillin, 12.5 μg/ml tetracycline (if appropriate), and 0.4 mM IPTG. Worms were added to plates individually; alternatively, agar chunks containing many worms were added. RNAi phenotypes were observ-

able within 1–5 days, depending on the target gene. Phenotypes were monitored in the F1 progeny of the animals placed on the plate. Plates contained sufficient quantities of bacteria to support the nematode growth during the course of the experiments, which lasted from one to five days. Freshly seeded plates were used in all the experiments described here; however, seeded plates that were stored for as long as two weeks at 4°C also produced RNAi phenotypes. For long-term maintenance of animals on dsRNA expressing food, worms from successive generations were transferred to fresh plates. At no time during dsRNA administration were the animals depleted of food.

2.5. Quantitation of dsRNA produced in bacteria

Cell culture: Competent cells were prepared using standard $CaCl_2$ methodology and were transformed with plasmid pLT61 or pLT76. Single colonies were inoculated into $2 \times YT$ media containing appropriate antibiotic and cultured overnight. The bacterial cultures were then diluted 1:500 in $2 \times YT +$ antibiotic(s) and were incubated until all samples reached $OD_{600} = 0.4$. (Faster growing cultures were periodically diluted so that all cultures were equally turbid at time of induction as quantitated by OD_{600}.) T7 polymerase was induced by the addition of 0.4 mM IPTG and the cells were cultured for an additional 4 h. Final cell numbers for each sample were calculated based on OD_{600}.

Analysis of dsRNA produced in bacterial strains: Total nucleic acids were extracted by lysing cell pellets (above) in 1 M ammonium acetate/10 mM EDTA plus phenol:chloroform at 65°C; precipitating nucleic acids using ethanol; removing DNA with DNAse I; and removing single-stranded RNA with bovine pancreatic RNase in 25 mM Tris, pH 7.5/50 mM NaCl/5 mM $MgCl_2$/0.5 mM dithioerythritol. The nucleic acid pellets were resuspended in 10 mM Tris/1 mM EDTA, pH 7.5 and loaded onto a 1% agarose/TAE gel, stained with ethidium bromide, and photographed. Sample volumes were normalized with respect to cell number after IPTG induction (calculated from OD_{600} readings of induced cells in culture). Approximately 10^9 cell equivalents were loaded onto each lane of the gel.

3. Results and discussion

3.1. Assays for bacteria-induced RNA interference

In each experiment, an engineered bacterial strain was provided as the sole source of food for *C. elegans*. The strains had been transformed with a single plasmid designed to express a specific segment of dsRNA. The bacterial food was provided to worms on standard nematode culture plates (+ additives) (Brenner, 1974). Interference phenotypes were monitored in the F1 progeny of animals placed on the modified food. In general, the levels of interference obtained with modified bacterial strains were compared to the levels seen using the original BL21(DE3) strain

(Timmons and Fire, 1998). Descriptions of plasmids and bacterial genotypes are provided in Section 2. To simplify the descriptions of the bacterial strains used in the feeding protocols, we have employed the following terminology for this paper: HT115–an RNaseIII mutant bacterial host; HT115(DE3)–host strain harboring a λDE3 lysogen (source of T7 polymerase); HT115(DE3)(L4417)–lysogenic strain harboring a plasmid capable of expressing dsRNA.

Two plasmid configurations utilizing the T7 promoter have proven useful in generating RNAi phenotypes (Timmons and Fire, 1998). (i) A bi-directional promoter configuration consists of two T7 promoters flanking a single
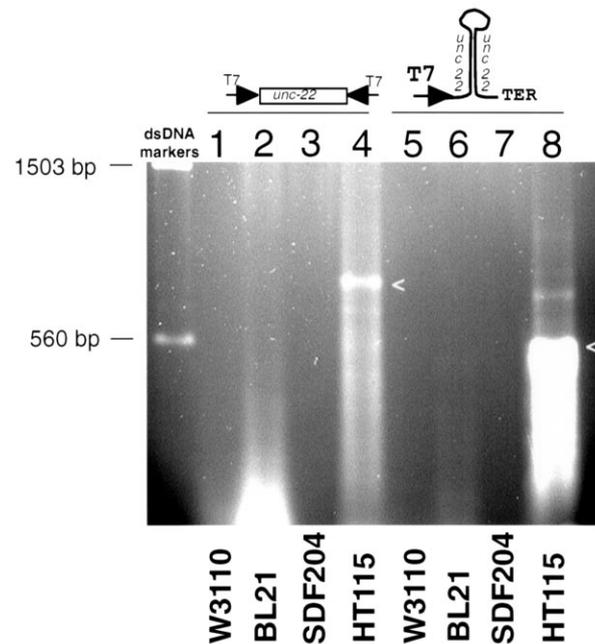

Fig. 1. Quantification of dsRNA produced in different bacterial strains. Bacteria of the indicated genotypes were lysogenized with λDE3, transformed with plasmids designed to express *unc-22* dsRNA, grown in liquid media, induced with IPTG, and processed for total nucleic acid (see Section 2). Following treatment with RNaseA (to remove single stranded RNA), samples were resolved by electrophoresis on agarose, and nucleic acid was visualized by staining with ethidium bromide. Top: Diagram showing two distinct plasmid configurations. Lanes 1–4: cells harbored pLT61, which contains an 887 base segment (composed of *unc-22* and polylinker sequences) flanked on each side by convergently oriented T7 promoters. Lanes 5–8: cells harbored pLT76, which contains a single T7 promoter driving a hairpin segment consisting of a 624 base stem (composed of *unc-22* sequences), an 864 base loop (composed of *gfp* and *unc-22* sequences), and a transcriptional terminator sequence 'TER' from T7 gene 10B (Studier et al., 1990). Sample volumes were normalized with respect to cell number. (Similar results were obtained with samples normalized with respect to induction volume.) The *rnc* alleles used are: BL21: *rnc*+; W3110: *rnc*+; SDF204: *rnc*+; HT115: *rnc*−. Substantially greater levels of dsRNA were produced from the HT115(DE3) (*rnc*−) host. Arrows indicate the major RNase/DNase resistant bands (presumed dsRNA). DNA size markers are shown at the left (slight differences in migration between equivalently sized dsDNA and dsRNA on these gels have been observed; LT and AF, unpublished). The dsRNA isolated from HT115(DE3) cells was also biologically active for RNAi; i.e. injection of this dsRNA into N2 worms produced a twitching phenotype (data not shown).

copy of a segment from the target coding sequence, and (ii) A hairpin configuration consists of a single T7 promoter driving an inverted repeat structure that has been interrupted by a non-homologous spacer DNA. These configurations are summarized in Fig. 1 (top).

3.2. Improved effectiveness of bacteria-induced RNAi using modified bacterial strains

Escherichia coli strain BL21(DE3), used in the initial development of bacteria-induced RNAi (Timmons and Fire, 1998), was originally chosen to take advantage of an IPTG-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage λDE3 (Studier et al., 1990; Boyer and Roulland-Dussoix, 1969). The BL21 host had been optimized for over-production of recombinant proteins (Studier et al., 1990; Tabor and Richardson, 1985; Wood, 1966). For example BL21 is deficient in at least two bacterial proteases: Lon and OmpT (Wood, 1966; Phillips et al., 1984; Grodberg and Dunn, 1988). We thus had no a priori reason to assume that BL21 might be optimal for RNA production. Furthermore, we considered that the protease deficiency, and perhaps other properties of this strain, might render it sub-optimal for over-production of dsRNA. We therefore tested several readily available *E. coli* strains not deficient in the Lon and OmpT proteases for interference (DH5α', TG1, SCS110, XL1-Blue MRF', W3110, and HB101). In each case, we made the strain lysogenic for λDE3, and then transformed with a plasmid (L4417) designed to produce *gfp* dsRNA. Although these strains yielded comparable degrees of *gfp* interference to that seen with BL21(DE3)(L4417), we observed slightly improved interference in some experiments with the bacterial strain W3110(DE3)(L4417). Subsequent optimizations have made use of strains derived from W3110.

We reasoned that the yield of interfering RNA might be improved if degradation of dsRNA in the bacterial host could be minimized. We further reasoned that this might be accomplished by removing (by genetic mutation) double-strand-specific RNases from the bacterial cell. A dsRNA-specific endonuclease (RNaseIII) is encoded by the bacterial *rnc* gene, and mutations in this gene are available. RNaseIII plays a prominent role in the processing and degradation of a subset of bacterial RNAs (Dasgupta et al., 1998; Takiff et al., 1989; Crouch, 1974; Gegenheimer and Apirion, 1981). A major role of bacterial RNaseIII is to initiate the maturation of rRNA from the 30S precursor RNA (Bram et al., 1980; Sirdeshmukh and Schlessinger, 1985). The enzyme has also been implicated in the post-transcriptional processing of approximately 10% of endogenous cellular mRNAs (Dennis, 1984; King et al., 1986; Portier et al., 1987; Takata et al., 1987; Talkad et al., 1978), of transgenic over-expressed mRNAs (Gitelman and Apirion, 1980), and of transcripts from bacteriophages T7, T3, and lambda (Dunn and Studier, 1973; Dunn and Studier,

1975; Hughes et al., 1987; Gottesman et al., 1982; Guarneros et al., 1982; Houlberg et al., 1983; Hyman and Honigman, 1986). In addition, RNaseIII has positive and negative effects on the translational efficiency of specific mRNAs (a) by inducing conformational alterations through site-specific cleavages, (b) by altering the stability of certain mRNAs, and (c) by removing sequences that stimulate or inhibit ribosome binding (Portier et al., 1987; Takata et al., 1987; Dunn and Studier, 1975; Court, 1993). Despite the relative importance of the reactions catalyzed by RNaseIII, null mutations in *rnc* are compatible with growth (Takiff et al., 1989).

We tested whether bacterial RNaseIII activity affects bacteria-induced RNAi by using a congenic set of bacterial strains, each with a different mutation in *rnc*: HT115 carries an *rnc* null mutation, and SDF204 is a control strain containing a wild-type *rnc* allele (Dasgupta et al., 1998; Takiff et al., 1989). These strains were derived from W3110. We made each strain lysogenic for λDE3, and these lysogenic host strains were then transformed with plasmids designed to produce dsRNA corresponding to *gfp* or to endogenous *C. elegans* genes. The dsRNA was produced from both plasmid configurations described previously (Section 3.1): double promoter and hairpin configurations.

The dsRNA produced in these bacteria was analyzed by preparation of total RNA followed by treatment with pancreatic RNaseA to remove single-stranded material. We found that the rnc^- cells [HT115(DE3)(pLT61) or HT115(DE3)(pLT76)] were most effective in accumulating extended dsRNA duplexes, while rnc^+ strains produced variable amounts of lower molecular weight dsRNAs (Fig. 1).

Fig. 2 shows the relative effectiveness of each strain in mediating RNAi against a *gfp* transgene. We define full interference as elimination of GFP fluorescence from >95% of cells in *gfp* transgenic animals. (This level of interference is observed in progeny animals after injection of *gfp* dsRNA into *myo-3::gfp* adults (Fire et al., 1998)). When *myo-3::gfp* transgenic animals were raised on BL21(DE3)(L4417), BL21(DE3)(L4431), or W3110(DE3)(L4417) bacteria, fewer than 15% of the adults in the population exhibited full interference (Fig. 2A,G,C). In contrast, >98% of the adult progeny of this transgenic worm strain showed full interference when reared on rnc^- strains HT115(DE3)(L4417) and HT115(DE3)(L4431) (Fig. 2E,I). This RNAi phenotype was dependent on T7 polymerase expression in the bacterial cell, as animals fed HT115(L4417) cells lacking the λDE3 lysogen showed no loss of GFP fluorescence (Fig. 2B). Bacterial strains SDF204(DE3)(L4417) and SDF204(DE3)(4431) were also less effective in the feeding protocol: most of the animals did not have a reduction in overall fluorescence intensity, and only a few animals had some muscle nuclei with no GFP expression (Fig. 2D,H).

The relative effectiveness of the modified feeding protocol was quantified by counting the residual, unaffected,
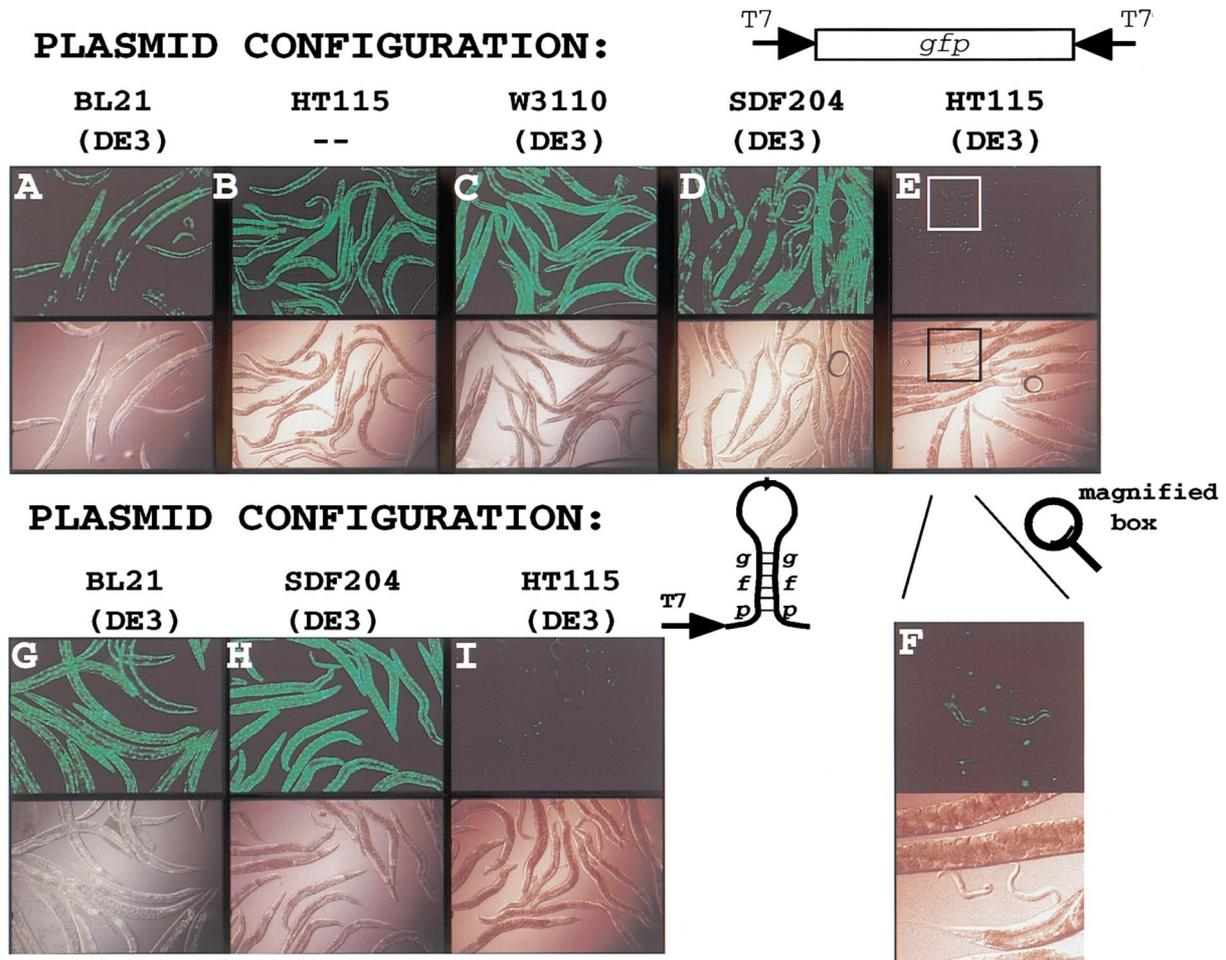
Fig. 2. Effectiveness of bacteria-induced RNAi on a *gfp* transgene. *myo-3::gfp* transgenic animals (PD4251) were placed as L3 larvae on the indicated bacteria and cultured at 20°C until F1 progeny reached adulthood. A–F: fluorescence and Nomarski images of animals fed bacteria harboring plasmid L4417 in the configuration depicted. G–I: fluorescence and Nomarski images of animals fed bacteria harboring plasmid L4431 in the configuration depicted. Five different bacterial hosts were used and are listed above each set of photos. Note that young larvae in this experiment are less affected by dsRNA introduced using this method than are their older siblings (F, magnified portion of panel E). A dramatic reduction in GFP fluorescence was observed in animals fed *rnc*[−] strains.
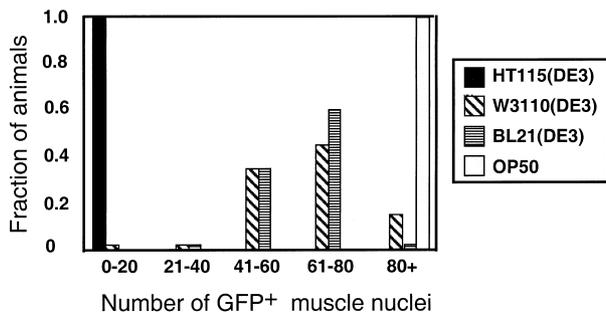

Fig. 3. Quantitation of the effectiveness of bacteria-induced RNAi. *myo-3::gfp* transgenic animals (PD4251) were placed as L3 larvae on the indicated bacterial hosts expressing *gfp* dsRNA from plasmid L4417 and cultured at 20°C until F1 progeny reached adulthood. (OP50 is the normal food source for *C. elegans* and does not contain a T7 polymerase.) Forty animals from each experiment were mounted for fluorescence microscopy, and all GFP[+] nuclei in each animal were scored. A dramatic reduction in both the number and fluorescence intensity of GFP[+] nuclei was observed in animals fed HT115(DE3)(L4417).

GFP^{+} cells in *myo-3::gfp* animals that had been fed either *rnc*[+] or *rnc*[−] strains (Fig. 3). In this experiment, none of the animals raised on BL21(DE3)(L4417) bacteria were fully affected. This *rnc*[+] strain produced no more than a 60% reduction in the number of GFP^{+} cells for any animal examined, and no animal had lost GFP fluorescence in all cells. In contrast, all of the animals fed the *rnc*[−] strain were severely affected: six percent of the HT115(DE3)(L4417)-fed animals had no GFP^{+} cells; 80% had five or fewer GFP^{+} cells per animal; and the remaining 14% had between five and 17 unaffected, GFP^{+} cells per animal. The fluorescence intensity in these residual GFP^{+} cells of animals fed HT115(DE3)(L4417) was much reduced in comparison to that seen in BL21(DE3)(L4417)-fed animals. Interestingly, the GFP^{+} cells in HT115(DE3)(L4417)-fed animals were almost exclusively found in the extreme anterior or posterior ends of the animals.

A marked improvement in the RNAi response using an *rnc*[−] host strain was also seen with endogenous *C. elegans*

% ANIMALS WITH STRONG TWITCHING PHENOTYPE						
plasmid configuration	plasmid name	BL21 (DE3)	HT115 (DE3)	HT115	SDF204 (DE3)	W3110 (DE3)
T7 *unc-22* T7	pLT61	0	100	0	24	12
T7 *unc-22* T3	L4366	–	6	–	0	–
T7 *unc-22* TER	pLT76	0	98	0	18	6
T7 *unc-22* T3	L4400	0	100	2	26	–

Fig. 4. Effectiveness of bacteria-induced RNAi on an endogenous gene. Wild-type (N2) animals from recently starved populations were placed onto plates seeded with bacteria expressing *unc-22* dsRNA derived from a plasmid in the configuration indicated (left column). Bacterial strains used are indicated at the top of the figure. Twitching phenotypes were scored in L4 and adult progeny in the absence of levamisole and are reported as a percentage of the total number of animals scored. As previously described for BL21(DE3) (Timmons and Fire, 1998), a weaker phenotype (twitching in the presence of levamisole) was observed in many of the remaining 'unaffected' animals. Plasmids used in the experiments are indicated in the second column of the figure. The T7 gene 10B transcriptional terminator is indicated by 'TER'. Plasmid promoters are indicated by arrows: triangular arrow = bacteriophage T7 promoter; flat arrow = bacteriophage T3 promoter (note that these bacteria have no source of T3 RNA polymerase, thus no activity from the T3 promoter would be expected in these cells). At least 200 animals were tallied in each experiment.

genes (Figs. 4, 5, and 6). The gene *unc-22* provides a particularly useful assay for the effectiveness of genetic interference, since phenotypes resulting from partial and severe reductions in function can easily be distinguished. A severe reduction in function results in a strong twitching phenotype visible under standard conditions, whereas a partial reduction in function [1.5–3-fold reduction in gene activity] results in a weak twitching phenotype visible only in the presence of the acetylcholine agonist levamisole (Fire et al., 1998; Brenner, 1974; Moerman and Baillie, 1979). Using the *rnc*+ strain BL21(DE3)(pLT61), we had obtained only a partial loss-of-function phenotype–85% of adult animals twitched in levamisole and no animals twitched in the

INTERFERENCE PHENOTYPES FROM FEEDING							
plasmid configuration	plasmid name	STRONG(%) BL21 (DE3)	STRONG(%) HT115 (DE3)	INTERMEDIATE(%) BL21 (DE3)	INTERMEDIATE(%) HT115 (DE3)	NONE(%) BL21 (DE3)	NONE(%) HT115 (DE3)
T7 *unc-54* T7	pLT62	0	44	0	50	100	6
T7 *fem-1* T7	pLT63	45	88	NA	NA	55	12
T7 *hlh-1* T7	pLT64	0	2	0	15	0	83

Fig. 5. Effectiveness of bacteria-induced RNAi in generating Unc-54, Fem-1, and Hlh-1 phenotypes. Wild-type N2 animals from a recently starved plate were fed bacterial cells harboring a dsRNA-expressing plasmid. (The bacterial strains used are indicated in the top row; the plasmid configuration and *C. elegans* gene insertions are indicated in the left column.) Plasmids used in the experiments are indicated in the second column. At least 200 animals were scored in each experiment. The phenotypes were scored in both adult and L4 progeny of the initially transferred animals.

PLASMID CONFIGURATION		-- +IPTG	AMP +IPTG	TET +IPTG	AMP+TET +IPTG	AMP+TET --
		% ANIMALS WITH STRONG TWITCHING				
T7 *unc-22* T7	TRIAL 1	23%	44%	66%	100%	99%
	TRIAL 2	68%	85%	84%	--	100%
	TRIAL 3	58%	64%	88%	100%	100%
T7 *unc-22* TER	TRIAL 1	81%	75%	64%	100%	100%
	TRIAL 2	42%	68%	73%	100%	100%
	TRIAL 3	77%	90%	91%	100%	100%

Fig. 6. Improved effectiveness of feeding protocol by inclusion of antibiotics. Wild-type worms were fed HT115(DE3) bacteria harboring either *unc-22* dsRNA expressing plasmids pLT61 (first set of experiments) or pLT76 (bottom set of experiments). The cells were cultured, induced, and plated in the presence or absence of the additives listed in the top line of the figure. Additives were included in the media and culture plates at the concentrations described in Section 2. Twitching phenotypes were scored in the absence of levamisole in adult and L4 progeny. *n* = 200 for Trial 1; *n* = 100 for Trials 2 and 3.

absence of levamisole (Timmons and Fire, 1998; Fig. 4). With W3110(DE3)(pLT61) or W3110(DE3)(pLT76) strains, we saw strong twitching phenotypes in no more than 12% of animals (Fig. 4). The *rnc*− bacterial strains HT115(DE3)(pLT61) and HT115(DE3)(pLT76) were most effective at inducing RNAi, producing strong phenotypes in 100% of progeny (Fig. 4). Again the SDF204(DE3) strain was less effective in inducing twitching with any of the plasmid configurations tested: fewer animals were affected and the number of affected animals varied from experiment to experiment. The Unc-22 phenotype was dependent upon a source of T7 polymerase in the bacterial cell, as the HT115(pLT61), HT115(pLT76), and HT115(4400) strains with no λDE3 lysogen and the HT115(DE3)(L4366) strain with a T7 polymerase source but only one T7 promoter site in the plasmid produced few affected animals.

rnc− mutant bacteria also provided increased effectiveness of RNAi in the feeding protocol for the target genes *unc-54*, *hlh-1*, and *fem-1* (Fig. 5). In the case of *unc-54*, no phenotype was ever observed when *rnc*+ host cells were employed. In contrast, use of the *rnc*− strain HT115(DE3)(pLT62) in the feeding protocol gave reproducible RNAi phenotypes. The Unc-54 phenotypes produced can be divided into three categories: strong (phenotypes similar to the null *unc-54* phenotype–complete paralysis and egg-laying defects (Brenner, 1974)); intermediate (phenotypes similar to strong hypomorphic *unc-54* mutants–incomplete paralysis with severe movement defects (Wills et al., 1983)); and no phenotype (wild-type movement). Approximately half the affected progeny fed HT115(DE3)(pLT62) exhibited a strong phenotype as adults, while a similar number of adults exhibited an intermediate phenotype (Fig. 5).

hlh-1 is a gene whose function is required during late embryonic stages (Chen et al., 1994). Injection of dsRNA into adults produces a strong loss-of-function phenotype in a majority of progeny (Fire et al., 1998). No phenotypically

affected animals were obtained following growth on BL21(DE3)(pLT64) cells, while phenotypes were observed using HT115(DE3)(pLT64) cells (Fig. 5). The phenotypes induced by feeding can be divided into three categories: strong (Lumpy/Dumpy appearance in early larvae–indicative of loss-of-function for *hlh-1*); intermediate (Lumpy or Dumpy appearance in larvae or adults–indicative of partial loss-of-function of *hlh-1*); and no phenotypes (wild-type appearance). A modest number of animals with strong and intermediate phenotypes and a large number of unaffected animals were obtained (Fig. 5). Affected progeny of animals fed HT115(DE3)(pLT64) arose at a much lower frequency (17%) than has been observed from standard genetic mutants or from injection of dsRNA into the parental germline (essentially 100%).

In contrast to the somatically active genes described above, the *fem-1* gene is required for proper germline development (Doniach and Hodgkin, 1984). Current models for sex determination suggest that this gene is required autonomously in the germline (Kuwabara et al., 1998; Mehra et al., 1999). Loss-of-function *fem-1* animals produce no sperm; therefore, the animals lack fertilized embryos and produce only oocytes. As with the somatically expressed genes described above, greater numbers of RNAi-affected animals were obtained in feeding experiments using the rnc^- bacteria expressing *fem-1* dsRNA (Fig. 5). A fraction of Fem-1 phenocopies were observed from feeding BL21(DE3)(pLT63) (45%); however, the numbers of affected animals increased significantly when the HT115(DE3)(pLT63) strain was used (88%) (Fig. 5). The fact that a significant fraction of animals is affected in the $rnc+$ strain is probably a reflection of the sensitivity of this gene to RNAi; however, we cannot rule out the possibility that this and perhaps other dsRNAs might be more resistant to bacterial RNaseIII.

As shown in Figs. 2 and 4, increased interference in *rnc*-backgrounds was observed using both double promoter and hairpin configurations. Although the two configurations were equally effective in these examples after several days of feeding, hairpin configurations often induced RNAi phenotypes more quickly than a bi-directional configuration (data not shown). One interpretation of this observation is that double-strandedness may be more easily achieved when sense and anti-sense strands are covalently connected, as in the inverted repeat.

3.3. Effective feeding-based RNAi is dependent upon conditions for bacterial growth and dsRNA induction

We have tested for interference using a variety of different conditions for the growth and induction of dsRNA-producing bacteria. Although the optimal conditions are likely to depend on the interfering construct and the target gene, several relevant parameters are noted below.

We have found that continuous induction of T7 polymerase in the presence of plasmids capable of producing dsRNA conferred a selective growth disadvantage. It would thus be expected that procedures which allow extended periods of T7 polymerase induction would result in selection for cells that have lost the capacity to over-express dsRNA. Such selection could result in the loss of T7 RNA polymerase, reversion of the *rnc* mutation through loss of Tn10, or loss of the dsRNA-producing plasmid. These elements can be selected for by incorporation of antibiotics into the medium: tetracycline selects against revertant rnc^+ host cells, while ampicillin selects against cells that have lost the dsRNA-expressing plasmid. The addition of either antibiotic alone to the plates resulted only in slight improvements in the protocol. The combination of tetracycline + ampicillin consistently and markedly improved the results obtained (Fig. 6). The inclusion of antibiotics to the assay also serves to prevent contamination from extraneous bacteria–a factor that can negatively affect the outcome of the feeding experiments.

3.4. Reversibility of bacteria-mediated RNAi

We have observed that the RNAi phenotype depends upon the continuous presence of dsRNA-expressing bacteria on the plate. Animals that twitch due to ingestion of *unc-22* dsRNA-expressing bacteria produce progeny with normal movement when switched to normal food. Furthermore, animals switched to normal food as young larvae fail to maintain the twitching phenotype. Similar results (re-expression of silenced genes when transferred to normal food) have been seen in animals transgenic for *gfp* that were fed *gfp* dsRNA-expressing food. In addition, if all the *unc-22* dsRNA- or *gfp* dsRNA-expressing food is depleted, most of the animals begin to lose the RNAi phenotypes.

3.5. Limitations of RNAi and comparisons of dsRNA delivery methods

For most genes, a reduction in mRNA levels to a critical threshold is required in order for a phenotype to be observed. The quantitative effectiveness of phenocopy production by RNAi for a given target gene, tissue, and developmental stage depends on a complex set of parameters that determine whether or not this critical threshold is achieved. Some of these parameters are likely to depend on the balance between dsRNA-mediated mRNA decay and *de novo* mRNA synthesis. First, degradation may be affected by tissue- and stage-specific aspects of dsRNA uptake and metabolism. Second, degradation may be affected by the availability of the RNAi machinery in a given tissue or at a particular developmental stage. Third, the natural stability of a target RNA has a significant effect on the potency of RNAi: highly stable RNAs provide a more susceptible target for gene-specific mRNA decay, whereas an mRNA that is intrinsically unstable will be less affected (Fire, 1999). Finally, it is conceivable (although not yet demonstrated) that some mRNAs may be resistant to

RNAi by virtue of sequence composition, secondary structure, or sequestration within the cell.

Although the feeding protocol can be very effective in producing a decrease in gene activity in adults and early embryos, we have in general observed a less severe effectiveness in young larvae, even those larvae whose ancestors have been raised on dsRNA-expressing food for several generations. Plates of GFP-expressing animals (PD4251) grown on HT115(DE3)(L4417) bacteria contained large numbers of unaffected (GFP⁺) L1 and L2 larvae (Fig. 2F). Likewise, a stock of animals grown continuously on HT115(DE3)(pLT62) bacteria (expressing *unc-54* dsRNA) contained L1 and L2 larvae with near-normal movement. These observations are the converse of what is seen with injection of these dsRNAs into the parent: for both target genes, effective interference is observed in the resulting progeny at early larval stages, while adult progeny of injected animals show partial recovery of the targeted genes (Fire et al., 1998).

Delivery of dsRNA by injection provides a bolus of dsRNA to the pre-fertilized egg, some of which is distributed throughout the embryo. This deposited dsRNA perseveres through embryogenesis to produce interference at later larval stages–stages when the embryo is effectively isolated from the environment by the eggshell which is deposited after fertilization. We hypothesize that the loss of RNAi in adult progeny of injected animals is due to simple dilution of the dsRNA trigger. (Consistent with this hypothesis, interference is generally lost in the second generation after injection.) Delivery of dsRNA by feeding provides a more continuous delivery of smaller concentrations of dsRNA. However, the embryonic period may be temporarily isolated from dsRNA delivery because the eggshell is impermeable to dsRNA or because the animal is not feeding at this stage, and this could provide a respite from the effects of dsRNA. We hypothesize that lack of feeding-mediated interference at early larval stages is a consequence of the relative impermeability of the nematode eggshell.

Consistent with these hypotheses are two other instances in which interference in older worms is more readily observed from dsRNA introduced by feeding than from dsRNA introduced by injection. In the PD4251 *myo-3::gfp* strain, vulval muscle *gfp* expression is partially resistant to RNAi following parental injection (Fire et al., 1998); however, *gfp* expression in this tissue is not resistant to *gfp* dsRNA when introduced by feeding (Fig. 2E,F,I; Fig. 3, data not shown). Likewise, egg-laying defects are not generally observed in animals injected with *unc-54* dsRNA; however, after feeding animals *unc-54* dsRNA, progeny animals exhibit this phenotype.

We have observed that all tissues are not equally sensitive to dsRNA delivered by feeding. In particular, several groups making use of RNAi have observed partial or complete resistance of certain genes expressed in the mature nervous system (M. Nonet, pers. Comm.; J. Fleenor, M. Hsu and A. Fire, unpublished). This resistance to RNAi is independent of the method of dsRNA delivery, as delivery of dsRNA by injection, soaking, or feeding produces the same effects in the nervous system (Fig. 7). The fact that dsRNA can be delivered continuously to the worm through feeding argues that the resistance seen in the nervous system is not stage-
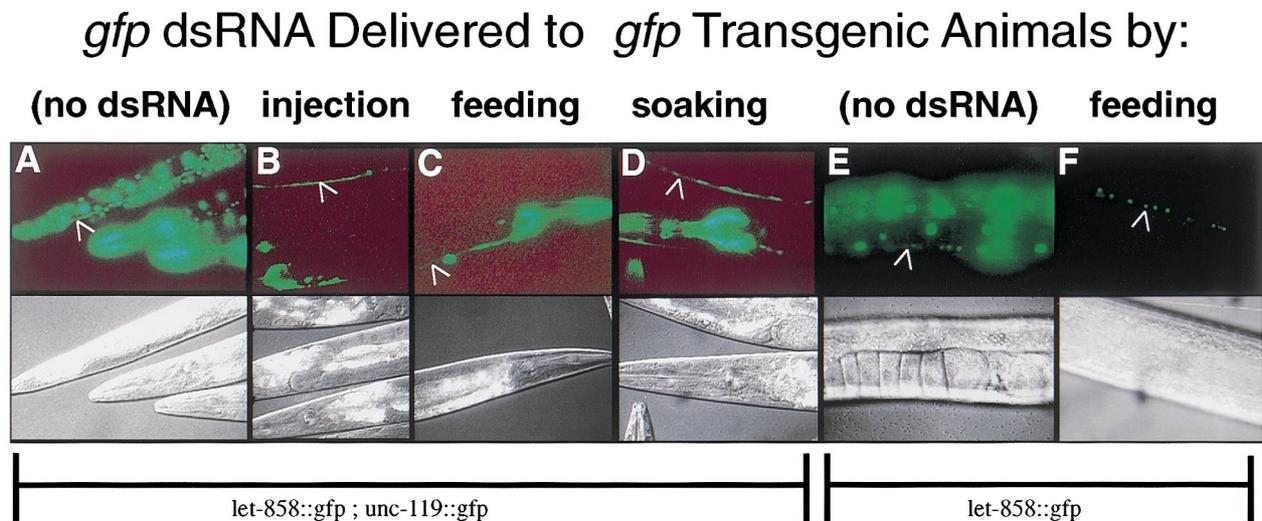

Fig. 7. Partial resistance to RNAi of some genes in the nervous system. Animals harboring two GFP-expressing transgenes were used: a *let-858::gfp* reporter transgene produces a nuclear-localized GFP-tagged protein that is expressed in all cells (Kelly et al., 1997), while an *unc-119::gfp* transgene produces a cytoplasmic GFP whose expression is limited to the nervous system (personal communications from Dr M. Maduro, Dr D. Pilgrim, and Dr H. Hutter). Panels A–D show animals carrying both transgenes. Panels E and F show animals carrying only the *let-858::gfp* transgene. Panel B shows progeny of animals injected with *gfp* dsRNA. Panels C and F show progeny of animals raised at 20°C on HT115(DE3)(L4417) bacteria expressing *gfp* dsRNA. Panel D shows animals soaked as L1/L2 larvae in *gfp* dsRNA and subsequently analyzed as adults for GFP fluorescence (Tabara et al., 1998). Note the persistence of GFP in the ventral nerve cord system in panels B, C, D, and F (arrows).

specific. Rather, the lack of effectiveness of dsRNA in the nervous system may be due to limiting components of the RNAi mechanism or to a factor destabilizing dsRNA in this tissue.

An additional difference in RNAi susceptibility by feeding is observed with respect to gender. During the course of our studies, we have noted that males appear to be less affected by dsRNA delivered through feeding. Males are slower to exhibit the RNAi phenotype than hermaphrodites: at 20°C, wild-type males exhibit the *unc-22* phenotype 3–5 days later than sibling hermaphrodites. In addition, several *him* strains were tested and we noted that these mutant males were also less affected than their hermaphrodite siblings. The explanation for these results may be trivial: perhaps males simply eat less food. A more complicated explanation may reflect a fundamental difference between the genders relative to components of the RNAi mechanism or to dsRNA uptake.

These observations argue that bacteria-induced RNAi and microinjection of dsRNA will have distinctive applications for production of mutant phenocopies.

3.6. Consequences of long-term feeding

Wild-type N2 worms and worms expressing different GFP-expressing transgenes (PD4251 and PD8047) have been reared on dsRNA-expressing food for over 20 generations. No loss of viability or morphological phenotypes were observed (other than gene-specific RNAi). Thus we have not observed any kind of response in worms that could be classified as a general 'panic response' against dsRNA.

4. Conclusions

We have shown that continuous feeding of *C. elegans* with bacteria that have been engineered to produce high levels of dsRNA can yield animals with loss-of-function phenotypes for a variety of target genes. Our data suggests, given several caveats, that an accurate indication of functional roles of genes for which loss-of-function mutations are not available can be achieved by comparisons of RNAi phenotypes from dsRNA injection and feeding techniques. We have observed specific limitations in RNA interference protocols (Fire et al., 1998; this work). These include lowered sensitivity in older larvae and adults (seen in the progeny of injected animals but not with continuous feeding), lowered sensitivity in late embryonic and early larval stages (seen with feeding but not injection), and lowered sensitivity in the mature nervous system (seen with RNA injection and feeding). The limitations in effectiveness of RNAi indicate substantial 'room for improvement' in these current protocols and reinforce the point that RNAi-based genetic interference can augment (but not substitute for) standard loss-of-function based mutational analyses.

Beyond the obvious applications to functional genomics, there are several implications for efficient feeding-based RNAi strategies. From a bioengineering perspective, it may be possible to use a bioengineered dsRNA comestible to modify gene expression or block viral infection in any organism which has a gene-specific dsRNA response similar to that of *C. elegans*. To respond to such an intervention, the target organism would need to have both a dsRNA-response mechanism (RNAi) and a dsRNA uptake/spreading mechanism that would produce responses such as those observed in *C. elegans*.

This work also raises the question of whether certain species might have evolved mechanisms that use stable dsRNAs to deter predatory attack. Further, such mechanisms might conceivably have been used to specifically modulate gene expression in members of the same species that cohabit the same area, or to modulate gene expression in different species that cohabit an area.

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