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Genome-wide RNAi screening in *Caenorhabditis elegans*

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Abstract

In *Caenorhabditis elegans*, introduction of double-stranded RNA (dsRNA) results in the specific inactivation of an endogenous gene with corresponding sequence; this technique is known as RNA interference (RNAi). It has previously been shown that RNAi can be performed by direct microinjection of dsRNA into adult hermaphrodite worms, by soaking worms in a solution of dsRNA, or by feeding worms *Escherichia coli* expressing target-gene dsRNA. We have developed a simple optimized protocol exploiting this third mode of dsRNA introduction, RNAi by feeding, which allows rapid and effective analysis of gene function in *C. elegans*. Furthermore, we have constructed a library of bacterial strains corresponding to roughly 86% of the estimated 19,000 predicted genes in *C. elegans*, and we have used it to perform genome-wide analyses of gene function. This library is publicly available, re-usable resource allowing for rapid large-scale RNAi experiments. We have used this library to perform genome-wide analyses of gene function in *C. elegans*. Here, we describe the protocols used for bacterial library construction and for high-throughput screening in *C. elegans* using RNAi by feeding.
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1. Introduction

Because of its anatomic and genetic simplicity, the nematode *Caenorhabditis elegans* has become an intensively studied simple animal model system. Furthermore, in 1998, *C. elegans* was the first multicellular eukaryote to have its genome completely sequenced, revealing 97 Mb of sequence encoding approximately 19,000 predicted genes [1]. The majority of these predicted genes remain uncharacterized; thus, although the genotype and phenotype of *C. elegans* have been studied in exquisite detail, the manner in which its genomic sequence is able to ultimately specify phenotype remains unclear. The study of genetics in *C. elegans*, from its inception as an experimental organism, has been largely based on classical forward genetics. Saturation forward-genetic screens have been extremely successful in comprehensively defining gene functions in organisms with compact genomes; however, this approach is less amenable to larger eukaryotic genomes since the sheer amount of time required to screen for and map every mutable gene is prohibitive. Completion of the *C. elegans* genome sequence, however, has greatly enhanced the prospect of employing reverse genetics as a complementary approach in the identification of gene function on a large scale.

Reverse genetics in *C. elegans* has been much more prevalent since 1998, when it was shown that the introduction of double-stranded RNA into a hermaphrodite worm results in potent and specific inactivation of an endogenous gene with corresponding sequence [2]. This technique, known as RNA interference (RNAi), enables rapid, targeted gene inactivation and has become an extremely important tool for studying gene function in vivo; moreover, because it is the simplest and quickest means of inactivating genes in *C. elegans*, RNAi has been rapidly embraced as a reverse-genetic tool and has dramatically accelerated the pace at which new gene functions are discovered. Initial studies of RNAi showed that injection of dsRNA into the head or tail of the animal was able to produce robust interference throughout the injected animal, including the germ line, suggesting that RNAi has the capacity to cross cellular boundaries. Indeed, subsequent studies demonstrated that RNAi could be performed in *C. elegans* simply by soaking worms in a solution of dsRNA [3] or by feeding them *Escherichia coli* expressing target-gene

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dsRNA [4] and that RNAi by feeding is an effective screening tool to determine the loss-of-function phenotype of a gene of interest [5,6].

All three methods (injection, soaking, and feeding) have been used effectively in large-scale RNAi screening [7–10]. Of these, RNAi by feeding has many advantages. First, because feeding is far less labor-intensive than microinjection, it is convenient for performing RNAi on a large number of worms or testing a large number of different genes. Second, feeding is considerably less expensive than either injection or soaking, which require the in vitro synthesis of dsRNA. However, RNAi by feeding suffers from one major drawback limiting its use as a high-throughput functional genomic tool: for each gene tested, it requires significant molecular biology work to clone a DNA fragment from the gene of interest into a special plasmid vector and then to transform it into a particular bacterial strain. To circumvent this limitation, we have constructed a bacterial library corresponding to roughly 86% of the estimated 19,000 predicted genes in *C. elegans* [7,11]. This library has already been screened for genes involved in a wide range of biological processes, including embryonic development [12], aging [13,14], fat regulation [15], and genome stability [16], and it should facilitate the ability of other investigators to perform their own genome-wide RNAi screens. Here we describe effective high-throughput methods for RNAi by feeding on a large scale. In addition, as the existing RNAi feeding library is not complete, we also describe methods for bacterial feeding library construction. Use of these methods and reagents should accelerate the identification of new gene functions in *C. elegans*.

2. Methods

2.1. Construction of RNAi feeding libraries

2.1.1. Background information

The sheer ease and speed of RNAi as an experimental technique have made it feasible to rapidly determine the loss-of-function phenotypes of large numbers of genes. Shortly after the *C. elegans* genome sequence became available, primers were designed that were capable of amplifying PCR products from each *C. elegans* predicted gene for use in generating DNA microarrays (S. Jones, personal communication); these primers (GenePairs) were synthesized and are commercially distributed by Research Genetics. GenePairs were optimized to PCR amplify products of roughly 1000–1500 bp and to have maximal overlap with predicted coding sequence; therefore, these primers were also ideal for performing RNAi of every predicted gene in *C. elegans*. Performing RNAi by feeding requires the cloning of a DNA fragment corresponding to a gene of interest into a vector for dsRNA expression. Therefore, in order to perform RNAi on the majority of the >19,000 predicted genes in the *C. elegans* genome by feeding, efficient methods had to be developed to allow rapid cloning of GenePair PCR products into an RNAi feeding vector. An overview of the cloning and screening is presented in Fig. 1.

2.1.2. Experimental procedure

Construction of bacterial RNAi feeding libraries required PCR amplification of DNA fragments from genes of interest, a method to efficiently clone these into a feeding vector, and a way to rapidly screen the resulting clones (overviewed in Figs. 1 and 2). Following is a detailed protocol for construction of RNAi feeding libraries for a large number of genes of interest.

We used the L4440 feeding vector as a basis for cloning PCR products ([4]; Fig. 1). This vector contains T7 promoter sites flanking each side of the multiple cloning site (MCS). After a gene-specific DNA fragment is cloned into this vector, dsRNA can be produced in bacteria by transcription with T7 polymerase. Plasmids were transformed into bacterial strain HT115(DE3), an RNase III-deficient strain of *E. coli* in which expression of T7 RNA polymerase is induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) [4,6]. The RNase III deficiency improves the efficacy of RNAi by feeding, presumably because the dsRNA produced is more stable in the bacteria [6].

Existing strategies for the direct cloning of PCR products take advantage of the terminal transferase activity of *Taq* DNA polymerase under standard PCR conditions, in which *Taq* preferentially adds an adenine (A) to the 3′ end of the product [17,18]. As a result, PCR products can be cloned directly into DNA vectors with complementary 3′ thymidine (T) overhangs (T-vector); this procedure is more generally referred to as "TA cloning" [19]. We tested several such strategies for efficient cloning of PCR products into the L4440 feeding vector; greatest success was achieved with an adaptation of a previously published method using terminal deoxynucleotidyl transferase (TdT) to catalyze the addition of a single T nucleotide to the blunt 3′ ends of the linearized vector (Fig. 2; [20]).

To adapt L4440 as a TA cloning vector, we used *Eco*RV to create blunt ends for 3′ T addition. The L4440 vector contains two *Eco*RV sites in the MCS, so we first modified the vector by digesting with *Eco*RV to release a small fragment and then recircularized the vector. This modified vector (L4440-RV) was then blunt-cut again using its single *Eco*RV site, and T-tailing was performed using TdT and 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP), thus ensuring 3′ addition of only a single dideoxynucleotide. PCR products could then be directly ligated into this vector due to the ability of *E. coli* to repair single-strand DNA breaks. Cloning efficiency was improved by self-ligation and gel purifi-

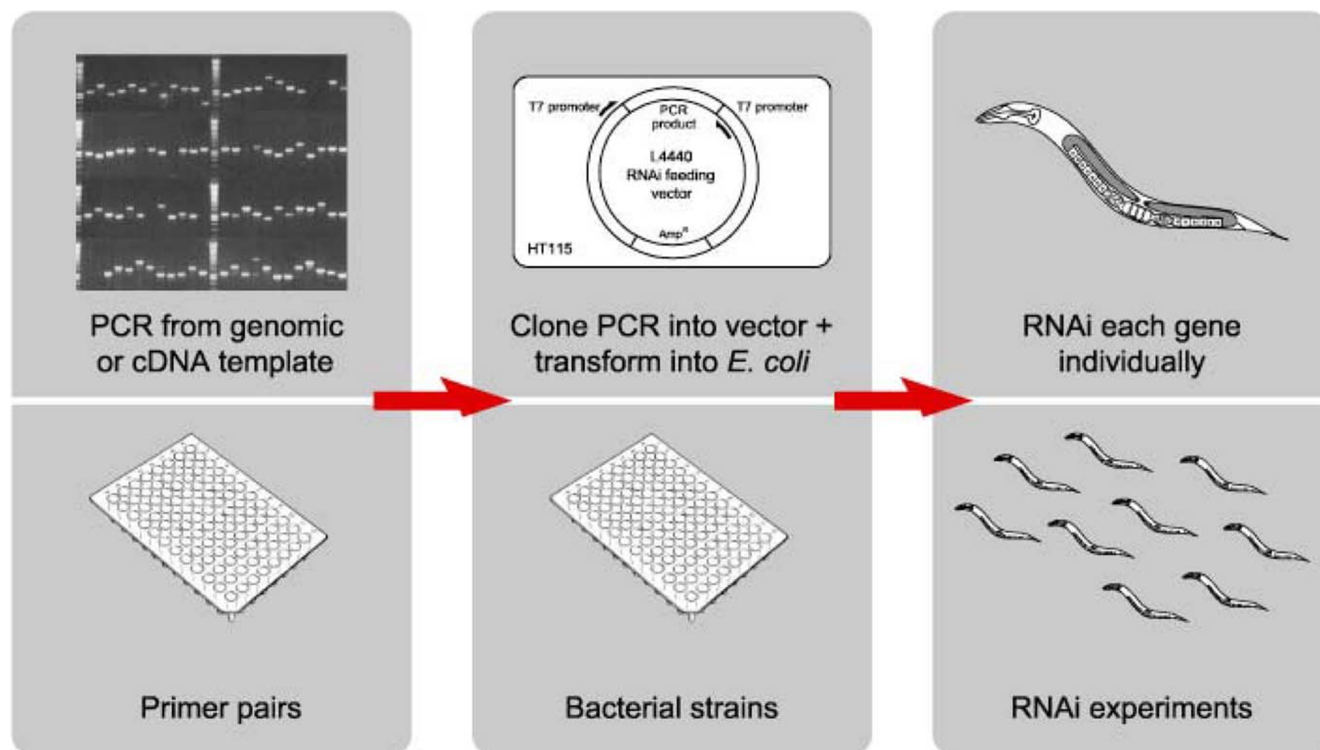

Fig. 1. Overview of screening strategy. Primers are used to PCR amplify the sequence of interest from genomic DNA or cDNA. The resulting PCR products are cloned into the L4440 RNAi feeding vector [4] and transformed into the HT115(DE3) bacterial strain [6], resulting in individual bacterial strains, each designed to target a single *C. elegans* predicted gene using RNAi by feeding. Each bacterial strain is then used to perform separate RNAi experiments to individually analyze the loss-of-function phenotype of each gene. Genes resulting in a phenotype are subsequently rescreened multiple times to ensure reproducibility of results.

cation to remove untailed vector prior to use in ligation reactions with PCR products. Because it was known that use of ethidium bromide and ultraviolet radiation for DNA visualization during the gel purification step could result in reduced cloning efficiencies, we employed an alternative method using crystal violet (CrVt) stain to visualize DNA; the use of crystal violet during gel purification resulted in ~8- to 10-fold more transformants [21].

We also optimized other parameters to increase the percentage of positive transformants. For example, following PCR amplification using the GenePairs primers, a prolonged 1-h final extension time increased the percentage of positive transformants, probably by increasing the percentage of A-tailed PCR products [22,23]. In addition, the HT115(DE3) bacterial strain proved difficult to make chemically competent by standard $CaCl_2$ methods; however, we found that adaptation of an alternative method routinely gave transformation efficiencies of $\sim 10^7$ transformants per microgram of vector [24,25].

Following are protocols developed for the creation of RNAi feeding libraries using the Research Genetics *C. elegans* GenePairs primer set (Figs. 1 and 3).

PCR from genomic DNA using GenePairs. C. elegans genomic DNA (gDNA) was prepared using standard methods [26]. For each GenePair, a separate PCR was performed using *Taq* DNA polymerase (Bioline): 2 µl (~50 pmol) of primers was added to 25 ng of gDNA, 1× Bioline NH_4 reaction buffer, 50 mM $MgCl_2$, 2 mM dNTPs (Boehringer), and 0.1 µl BioTaq (Bioline) in a 25-µl reaction. Preheating the PCR machines to 94 °C prior to cycling was found to improve the quality of the resulting PCR products; following 2 min at 94 °C, 35 cycles were performed at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, and then the reactions were incubated a final 1 h at 72 °C to improve A-tailing. PCRs were then stored at −20 °C until use.

TA cloning vector preparation. T-tailing of the modified L4440-RV feeding vector was performed as follows (based on the method first presented in [20]):

1. Twenty micrograms of L4440-RV vector were digested with 300 U of *Eco*RV (Boehringer) in a 900-µl reaction for 3 h at 37 °C.
2. The enzyme was heat-killed at 68 °C for 10 min, and the DNA was ethanol precipitated and resuspended in 34 µl of dH_2O.
3. Two such batches were combined into a single tube, and TdT reactions were carried out in 1× TdT buffer (Promega), 5 mM ddTTP (Pharmacia, Sigma, or MBI-Fermentas), and 30 U of TdT (Promega). TdT reactions were performed in a PCR machine held at

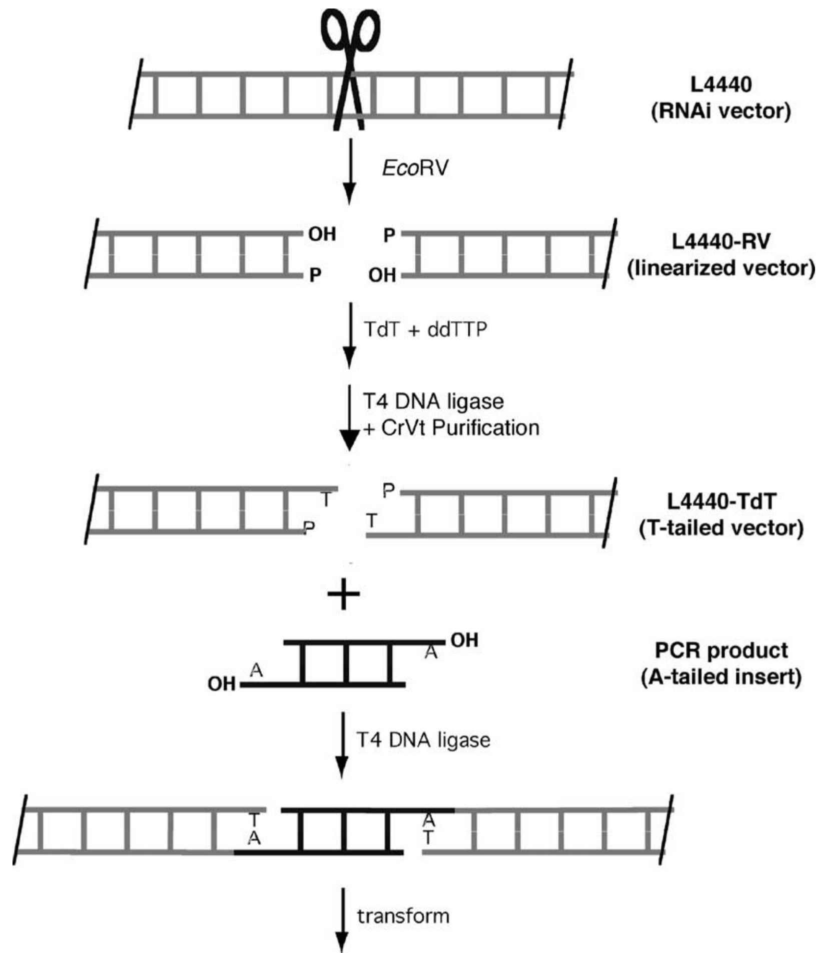

Fig. 2. Construction of TA cloning vector. The L4440 feeding vector [4] was blunt-cut with *Eco*RV and a small insert from the MCS was removed. A single T nucleotide is catalytically added to the 3′ ends of the linearized vector using terminal deoxynucleotidyl transferase (TdT) and ddTTP [20]. After autoligation, nontailed products are removed by gel purification using a crystal violet (CrVt) staining method [21]. PCR products made with a 1-h final extension (for enhanced A-tailing) are ligated to this purified T-tailed vector, and the resulting ligation products are transformed into the HT115(DE3) bacterial strain.

37 °C for 1 h to ensure temperature stability and to prevent evaporation.

4. Following heat-killing of the enzyme at 70 °C for 10 min, the resulting DNA was thoroughly cleaned using a Qiaex II DNA purification kit (Qiagen); clean DNA was eluted in 84 μl of dH_2O. (N.B. Care must be taken in this cleaning step, as mixture of any residual TdT buffer with ligase buffer will result in a change in color and formation of a precipitate that will interfere with subsequent ligation reactions.)
5. The vector was self-ligated to circularize untailed DNA material in a 100-μl reaction using 1× T4 DNA ligase buffer (Promega) and 15 U of T4 DNA ligase (Promega); reactions were incubated at 4 °C overnight (~12 h).
6. Self-ligation reactions were run on a CrVt-containing TAE gel as described below (~50 μl/lane); bands corresponding to linearized full-length L4440-RV were excised using a sterile blade, and the vector DNA was purified using a Qiagen gel extraction kit. Final vector DNA was eluted in 50 μl of 10 mM Tris–HCl, pH 8.5, per band and was stored at −20°C until use.

Crystal violet-stained agarose gels were prepared according to the protocol described [21]. Molecular-biology-grade agarose (Life Technologies or Helena Biosciences) was dissolved in 1× TAE (40 mM Tris–acetate, 1 mM EDTA, pH 7.2). After the mixture was allowed to cool, CrVt (Sigma) was added to a final concentration of 10 μg/ml before the gel was cast. Similarly, 10 μg/ml CrVt was added to 1× TAE buffer prior to running the gel. DNA samples were loaded using 2% Ficoll 400 plus 0.002% xylene cyanol; bromphenol blue cannot be used because it interacts with CrVt. Sufficient quantities of DNA (>1 μg) must be used for visualization with CrVt.

Ligation of PCR products into TA vector. The T-tailed vector from the above reactions was combined with PCR products in 96-well format to perform ligations in a 5-μl reaction: 2 μl (~100 ng) of PCR product was

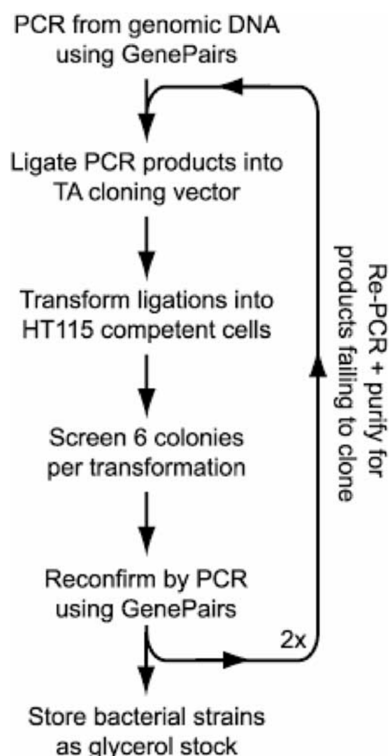

Fig. 3. High-throughput construction of RNAi feeding libraries. First, PCR is performed in 96-well format using desired primer pairs. These PCR products are ligated into the TA cloning vector in batches of 576 (6 × 96) and are transformed into the HT115(DE3) bacterial strain in 96-well format. Six colonies are screened per transformation by PCR using a T7 primer; positive transformants are confirmed by PCR using the original primer pairs. Multiple iterations of these methods are used to maximize the number of confirmed clones; after the first cloning attempt, PCR products are purified using guanidine–HCl to eliminate primer dimers, which is a major cause of background colonies during cloning. Confirmed clones are preserved as frozen glycerol stocks.

added to 0.5 μl (~25 ng) of vector with 1× T4 DNA ligase buffer (Promega) and 0.6 μl (roughly 1.5 U) of T4 DNA ligase (Promega). Ligations were incubated overnight (~12 h) at 4 °C.

Preparation and use of chemically competent HT115(DE3). Efficient chemically competent HT115(DE3) bacteria (~10^7 colony-forming units/μg DNA) were prepared according to a protocol based on the TSS (transformation and storage solution) method (first described in [24]):

1. A single colony of HT115(DE3) [4] was inoculated in LB medium and was incubated overnight (~12 h) with shaking at 37 °C. The HT115(DE3) genotype is F^-, mcrA, mcrB, IN(rrnD-rrnE)1, $lambda^-$, rnc14::Tn10 (DE3 lysogen: lacUV5 promoter–T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus). The Tn10 transposon interrupting the rnc14 gene carries a tetracycline-resistance gene. The strain was first streaked onto LB plates containing tetracycline to maintain the insertion into the RNase gene rnc14, but tetracycline was not included when making competent cells (loss of tetracycline resistance in the absence of selection was not observed in over 50,000 colonies tested).
2. The overnight culture was diluted 1:1000 into 400 ml fresh LB in a sterile flask and incubation was continued with shaking at 37 °C until the culture reached an OD_{600} of exactly 0.3 (roughly 2.5–3 h).
3. The culture was chilled in an ice-water bath for 10–15 min, and then 1 vol (400 ml) of ice-cold, sterile 2× TSS (20% w/v polyethylene glycol 3350, 100 mM $MgCl_2$ in LB plus 10% v/v dimethyl sulfoxide, pH 6.5) was added and was mixed gently; this mixture was incubated in the ice-water bath 15–20 min.
4. Cells were pelleted by centrifugation for 10 min at 1000*g* at 4 °C, the supernatant was discarded, and the pellet was resuspended in 0.1 vol ice-cold 1× TSS (2× TSS diluted 1:1 in sterile LB).
5. Roughly 100 μl of cells was aliquoted into each well of 96-well 1.2-ml deep-well plates (Eppendorf); plates were sealed tightly with the provided mats and were flash-frozen in liquid nitrogen. Cells were stored at −80 °C until use. (Since ~ 33μl of competent cells were used for each transformation reaction, each 96-well plate of cells was used for 3 × 96 transformations.)

For transformation reactions, ligations in 96-well format were incubated in an ice-water bath for 10 min, and then ~33 μl of competent cells was added to each well and incubated for ~15–20 min. Transformations were performed by heat shock at 42 °C for 1.5–2 min, and cells were allowed to recover in an ice-water bath for 5 min. Transformations were plated onto 6-well (Nunc) LB–agar plates with 50 μg/ml ampicillin (Amp) and spread using 2.5-mm glass beads (Philip Harris Scientific). After being dried at room temperature for ~30–60 min, the beads were discarded, and the plates were inverted and were incubated at 37 °C overnight (~18 h).

Transformant screening and library assembly. Following transformation, six bacterial colonies were screened by PCR from each ligation. Colonies were picked directly into PCR mix (1× Bioline NH_4 reaction buffer, 50 mM $MgCl_2$, 10 pmol T7 primer, 2 mM Boehringer dNTPs, 0.1 μl BioTaq) in 96-well-plate (Eppendorf) format. Prior to PCR, viable bacteria were replicated from these 96-well plates onto Omnitray flat plates (Nunc) containing LB–agar plus 50 μg/ml Amp and 15 μg/ml tetracycline (Tet) using a 96-pin replicator (Invitrogen); these were allowed to incubate overnight at 37 °C and were stored at 4 °C until use (for a maximum of 4–6 weeks). PCRs were typically performed in sets of 576 in 6 × 96-well PCR plates (i.e., corresponding to one 96-well plate of primers). The PCR machines were preheated to 94 °C prior to cycling; following 2 min at

94 °C, 25 cycles were performed at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s, followed by a final extension of 72 °C for 7 min. PCR products for which no positive bacterial colony was isolated were subjected to two further rounds of cloning. It was found that one reason for cloning failure was the presence of primer dimers; thus, in the third round, PCR products were purified using a Qiaquick 96 PCR purification kit (Qiagen) including guanidine–HCl washes to remove primers prior to ligation.

Clones that tested positive by PCR with T7 primer were manually regridded to correspond to the original 96-well GenePair format and were replicated onto LB–agar + Amp/Tet flat plates. These bacteria were then retested by PCR using the original GenePairs to confirm the identities of the inserts. Retesting clones was performed by PCR as described above, except that 2 μl of GenePairs was used instead of T7 primer; cycling conditions were likewise identical except that the annealing step was done at 58 °C (instead of 52 °C) for 30 s. Nonconfirmed clones were removed from flat plates by chunking out the bacteria using a sterile micropipette tip. Frozen glycerol stocks were made from confirmed clones by growing bacterial cultures to log phase (~4–6 h) and storing them at −80 °C in 15% glycerol in 96-well TC plates (Nunc) sealed tightly with PCR foil (Eppendorf).

These methods are useful for constructing bacterial feeding libraries corresponding to large numbers of specific genes of interest. We built a library containing 16,757 bacterial strains, which allows RNAi of ~86% of *C. elegans* genes [5,7]. This library is publicly available through MRC Gene Services (http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/Celegans.shtml) as frozen glycerol stocks arrayed in 384-well plates.

2.2. High-throughput RNAi by feeding

2.2.1. Background information

Timmons and Fire first described a method for RNAi in which bacteria expressing dsRNA are fed to *C. elegans* [4]. As discussed above, RNAi by feeding is performed using HT115(DE3) bacteria transformed with the L4440 vector containing a fragment corresponding to the target gene [6]. Induction of T7 polymerase expression drives transcription of the complementary DNA strands and thus generates dsRNA, which is absorbed by worms via the intestine following ingestion of bacteria and is subsequently distributed throughout the animal. Based on these initial discoveries, we developed an optimized protocol for RNAi by feeding by varying parameters that could affect the efficiency of RNAi [5].

2.2.2. Experimental procedure

The following sections describe an adaptation of the optimized protocol for RNAi by feeding [5] for high-throughput phenotypic screening in conjunction with bacterial feeding libraries (Figs. 4 and 5). In brief, L3–L4-stage hermaphrodites are fed for 3 days to allow the RNAi to take effect, and then three worms are cloned to individual wells for each gene screened. Each of these is

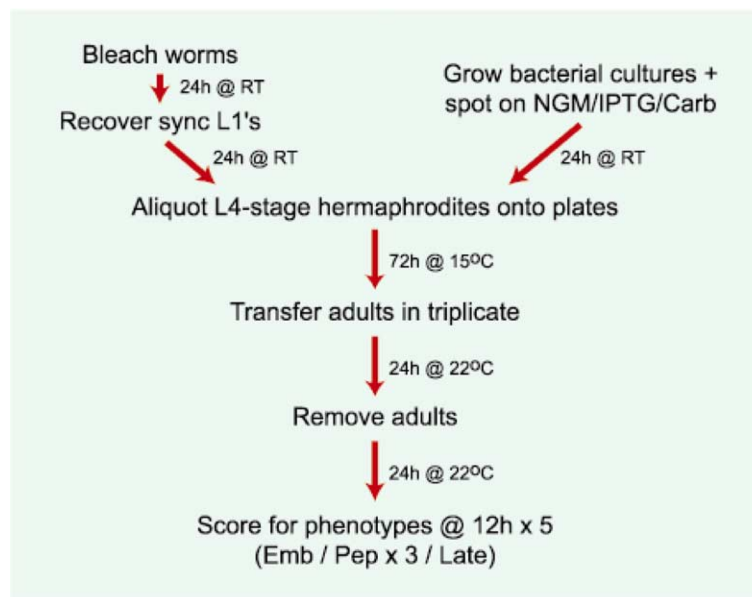

Fig. 4. Feeding schedule. Shown is a schematic for high-throughput RNAi by feeding. Worm preparation prior to aliquoting is performed concomitant with preparation of bacteria and RNAi feeding plates. The following abbreviations are used to described scoring tasks: scoring for embryonic lethal phenotypes (Emb), scoring for postembryonic phenotypes (Pep), scoring for late postembryonic or adult phenotypes [Late; e.g., egg-laying defective (Egl) or sterile progeny (Stp)].

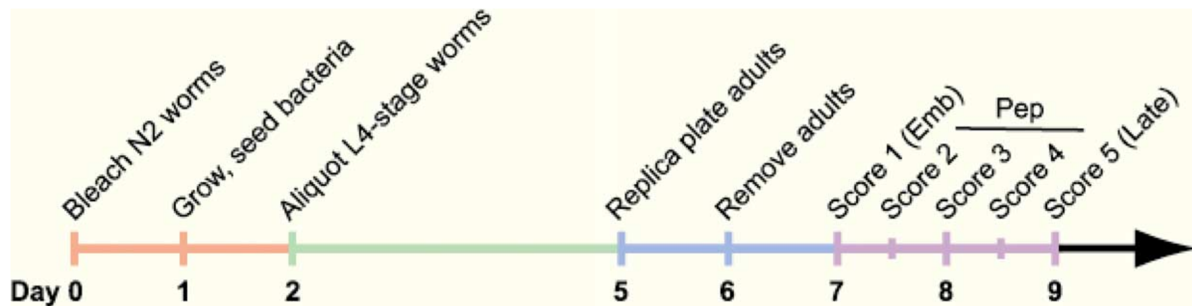

Fig. 5. Feeding time line. Shown is a schematic of the time frame of a single experiment using RNAi by feeding. Listed above the time line are specific tasks required for feeding experiments, and below the time line is the number of days after beginning an experiment that the given task is performed. Abbreviations are described in the legend to Fig. 4.

allowed to lay eggs for 1 day and then is removed, after which the progeny are scored for mutant phenotypes at five time points. Siblings of the cloned hermaphrodites and the progeny produced during the initial feeding period can also be scored for RNAi phenotypes.

This is a general screen for identifying a mutant phenotype in the fed worm or in its progeny, from embryogenesis through to adulthood. This can be modified for screens in which only a single scoring time is needed (expression of GFP at a particular stage, synthetic lethality, etc.). In particular, much time can be saved if the laborious step of cloning worms to individual wells can be avoided. This is only necessary if the number of worms will be too great for the amount of food or if some amount of synchronization is needed.

Bacterial preparation and induction. For feeding plates, NGM agar [26] was prepared including 25 μg/ml carbenicillin (carb) and 1 mM IPTG; 2.5 ml of agar was dispensed into each well of 12-well TC plates (Nunc). Plates were allowed to dry inverted for 4–8 days at room temperature before use. Using a 96-pin replicator (Invitrogen), bacteria from glycerol stocks were spotted onto LB–agar plates including 50 μg/ml Amp and 15 μg/ml Tet; this was done in Omnitray flat plates (Nunc). Using an eight-channel pipettor, large innocula of bacteria were picked, inoculated into LB with 50 μg/ml Amp, and grown for 6–8 h with shaking at 37 °C; 1 drop of this culture was seeded per well into the above NGM-derivative plates, 4 wells per gene (i.e., three genes per plate, one per row), and the plates were dried thoroughly before being incubated overnight (~12–24 h) at room temperature to allow the bacteria to grow and to begin induction.

Worm synchronization, aliquoting, and feeding. N2 worms were cultured on standard NGM plates with OP50 *E. coli* prior to use in feeding experiments. Plates with large numbers of eggs were washed with hypochlorite solution (1.5 ml of 4 N NaOH, 750 μl of Aldrich sodium hypochlorite solution, 7.5 ml of dH_2O); plates were also scraped with a glass rod to remove eggs stuck to the medium. The solution was transferred to Eppendorf tubes, which were shaken vigorously by hand for 3–5 min to homogenize large worm particles. Eggs were pelleted by gentle centrifugation (7000 rpm) and were washed 3× in M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M $MgSO_4$, in H_2O to 1 L). Eggs were incubated in M9 and were allowed to hatch overnight (~12 h) at room temperature. The resulting synchronized L1-stage worms were then put on standard NGM plates with OP50 and were allowed to develop to early L4 stage (~30 h at 25 °C). Worms were washed off the plates in M9 + 0.01% Triton X-100 (Sigma), pelleted gently by centrifugation as described above, and washed several times to eliminate residual OP50. Roughly 7–10 worms were then aliquoted into the first well of each row of each plate (corresponding to one well per gene on the plate). The plates were allowed to dry, and the worms were incubated for 72 h at 15 °C (or 36 h at 22 °C).

Worm handling and scoring. After the initial feeding period, three adult worms were transferred from the first well of each row into each of the other three wells seeded with the same bacterial strain (thereby performing each experiment in triplicate to ensure reproducibility of results). The plates were then returned to the selected feeding temperature, and the worms were allowed to lay eggs for 24 h and then removed by aspiration. After the plates were incubated a further 24 h at 22 °C to allow surviving eggs to hatch, embryonic lethality and early larval phenotypes could be scored using a dissecting microscope (Fig. 6). Postembryonic phenotypes were scored at four additional successive 12-h intervals. Progeny laid on the first well were also be scored for postembryonic phenotypes. Clones that resulted in a phenotype following RNAi by feeding were subsequently refed and reanalyzed for confirmation.

Streamlined screens. For many screens, it will be adequate to do all feeding and scoring of worms in the same well, which would significantly reduce screening time. A major factor to take into account is the amount of bacteria that will be needed to feed the number of worms that will be in the well. In the standard feeding protocol above, one well of a 12-well plate should have sufficient bacteria to feed the brood resulting from a

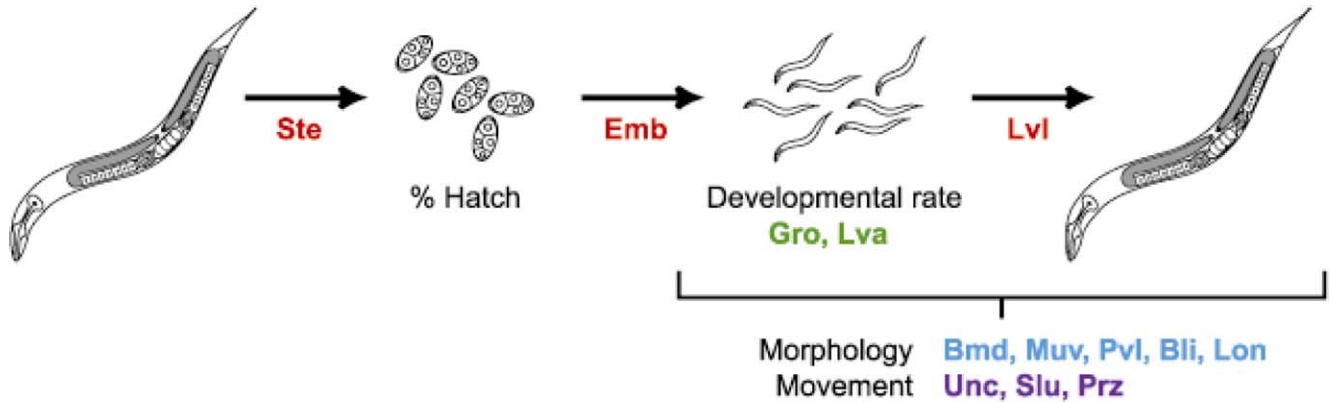

Fig. 6. Analysis of RNAi phenotypes. L3- or L4-stage hermaphrodite worms are allowed to feed on a specific bacterial strain for 72 h at 15 °C. Three (then adult) worms are transferred to separate wells inoculated with the same bacteria and allowed to lay eggs for 24 h at 22 °C, after which they are removed. After the plate is incubated for an additional 24 h, wells are scored for sterility (Ste; no eggs), embryonic lethality (Emb; dead eggs), slow postembryonic growth (Gro), or larval lethality (Lvl). At the end of the next four consecutive 12-h intervals, the progeny are scored for larval arrest (Lva) or Gro or for one of a number of postembryonic defects, including those affecting morphology or movement. Examples of these phenotypes are Bmd (gross body morphology defect), Muv (multivulva), Pvl (protruding vulva), Bli (blistering), Lon (long), Unc (uncoordinated), Slu (sluggish), and Prz (paralyzed).

24-h egg lay from one hermaphrodite. However, if several worms are left in a well for several days, then they and their progeny need to be fed, and larger 6-well plates seeded with several drops of bacterial culture will be required. In this case, progeny may show a range of RNAi phenotypes, with the most recently laid progeny being most affected. In some cases, this can be an advantage, as there will often be surviving progeny from clones that induce embryonic lethality, allowing later postembryonic functions to be assayed. Feeding in liquid culture is also feasible, which can be advantageous for certain applications (A. Fraser and J. Ahringer, unpublished).

3. Discussion

RNAi is a remarkably convenient method for determining the loss-of-function phenotype of an individual gene of interest. By performing RNAi on a large scale using a genome-wide RNAi library, this reverse-genetic method can essentially be used as a forward-genetic screening tool. In performing such a large-scale RNAi screen, several important factors must be considered. First, genes have different sensitivities to RNAi. Genes that encode proteins with long half-lives can be difficult to target, since RNAi results in message degradation and does not directly affect the protein [27,28]. In addition, tissues have differing sensitivities to RNAi, with the nervous system in particular being somewhat resistant [6,29]. This latter difficulty can be partially overcome by using an RNAi-supersensitive *C. elegans* strain (e.g., *rrf-3*; [30,31]). Second, RNAi phenotypes can sometimes be inconsistent from animal to animal or may be similar to a hypomorphic rather than a null allele. In some cases this can be an advantage, since a complete null may prevent evaulation of a late role in development (e.g., essential genes can be assayed for a role postembryonically if RNAi does not result in lethality in every individual). Third, any gene that has a significant amount of sequence identical to that of the introduced dsRNA will be silenced in the RNAi experiment (e.g., very close homologs often will be simultaneously inhibited). The exact level of identity with the introduced dsRNA needed for effective RNAi in vivo is not clear, but examination of a large number of experiments suggests that 80% identity over 200 bp will usually predict whether a gene will be silenced or not (data not shown). This means that it is not always possible to attribute a mutant phenotype to silencing of a particular gene; additional RNAi experiments targeting different regions of the gene can usually resolve this, as can the isolation of genetic mutants. In some cases, silencing of more than one gene can be an advantage, as genes with overlapping function would be difficult to identify in a traditional forward genetic screen in which only one gene would be mutated. Finally, to confirm the RNAi results, it is desirable to isolate a mutation in the gene using traditional reverse-genetic methods [32]. In cases in which a gene has multiple functions during development, a mutant may not be useful for confirming an RNAi phenotype (e.g., mutants that are homozygous sterile cannot be assayed for maternal effect embryonic lethality). In such cases, an antibody recognizing the encoded protein can be used to confirm that its abundance is significantly reduced after RNAi.

In conducting the general screen described here, roughly 200 genes per day can be screened by 3 people; this translates to screening the entire genome for a range of loss-of-function phenotypes in just over 3 months. In

such a screen, in which lethality and obvious developmental defects are scored, most of the labor is in the physical manipulation of worms (i.e., the transferring and removing steps) and the phenotypic scoring steps. Therefore, devising screens not requiring worm transfer (as would be the case in screens for most postembryonic phenotypes) would reduce the screening time significantly. Alternatively, the speed of scoring can be enhanced using efficient phenotypic assays (e.g., GFP-based screens or screens using an automated worm sorter). Feeding in liquid culture would also accelerate screening time. In theory, such screens could allow screening of >1000 genes per day, thus allowing genome-wide analysis in well under 1 month. Future screens carried out under different environmental conditions, in different genetic backgrounds, or by focusing on different phenotypes should rapidly aid in a fuller understanding of *C. elegans* biology. Furthermore, since over 60% of *C. elegans* genes have a homolog outside the worm, many of these advances should be broadly applicable to the study of other organisms.

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