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# A METHOD FOR THE ISOLATION OF LONGEVITY MUTANTS IN THE NEMATODE *CAENORHABDITIS ELEGANS* AND INITIAL RESULTS

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SUMMARY

The free-living nematode *Caenorhabditis elegans* is used as a genetically manipulable experimental system for the study of aging. Utilizing a temperature-sensitive sterile strain with a normal life span, a method is described for the isolation of mutant strains with significantly increased life spans. Eight mutant strains were isolated each having increased life spans. Two mutant strains were spontaneous dauer formers, accounting for their increased longevity. Another was chemotaxis-defective, causing reduced food intake which could account for its increased life span. Five mutants suffered from varying degrees of paralysis affecting their rate of pharyngeal pumping and food ingestion. The high correlation of the decreased rate of food ingestion of these mutants with their increased longevity is interpreted as indicating that the increased longevity is most likely due to reduced caloric intake. These results appear to indicate that specific life span genes are extremely rare or, alternatively, life span is controlled in a polygenic fashion.

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*Key words*: *C. elegans*; Life span; Mutants; Ageing; Genes

## INTRODUCTION

It is obvious from species-specific life spans and the inheritance of longevity in certain inbred strains that life span is genetically controlled [1]. Determination of the exact nature of this control can be facilitated by mutational analysis, that is, by the selection and analysis of life span mutants [2]. For example, if life span were controlled by specific aging genes that are activated at maturity and cause the subsequent senescence of the organism, then mutation in one or more of these genes would presumably lead to increased longevity [3]. Because many mutations

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in vital genes will lead to a decrease in life span, it is potentially more interesting to obtain mutants with significantly increased life spans. However, due to the difficulty of obtaining such mutant strains it has not been possible to ascertain directly the nature of the genetic control on longevity. We have recently been successful in devising a scheme for the isolation of mutant strains of the nematode *Caenorhabditis elegans* having significantly increased life spans.

*C. elegans* is a simple multicellular eucaryote whose genetics [4,5], development [6,7], and cellular anatomy [8–10] have been described in exquisite detail. Although this nematode is normally a self-fertilizing hermaphrodite (XX), males (XO) do arise by a process of nondisjunction of the X chromosomes [11], thereby allowing genetic manipulation. *C. elegans* has in recent years become an increasingly popular experimental system for the study of aging [12–14]. Because *C. elegans* is a genetically manipulable organism with a short generation time and life span [12], it is ideally suited for the mutational analysis of longevity. In this article we describe a method for the isolation of mutant strains of *C. elegans* that exhibit significantly increased life spans, and our initial characterization of these mutants.

## MATERIALS AND METHODS

### *Culture methods*

*Caenorhabditis elegans* var. *Bristol* was from the University of Houston stock. Strain designations are those of Horvitz *et al.* [15]. The mutant strain DH26 was obtained from the University of Colorado stock and carries a temperature-sensitive mutation causing a spermatogenesis defect leading to sterility at 25°C DH26 exhibits a wild type life span at all temperatures [12]. For life span studies worms were grown on NGM agar plates seeded with *Escherichia coli* OP50 [4]. At daily intervals worms were transferred to fresh plates with a fresh bacterial lawn and the percentage survival was recorded [12]. Pharyngeal pumping rates were determined by direct observation using a wild dissecting microscope (×50 magnification).

### *Synchronous cultures*

Synchronous cultures for life span determinations were obtained by the hatching method described previously [4,12]. In all experiments zero time was the time of hatching.

### *Labeling*

Determination of the amount of food ingested was done by feeding synchronous samples of worms *E. coli* that had been grown on $^{35}$S-sulfate as described previously [16]. After a 2-h feeding time the worms were washed extensively in M9 salt solution [4] and counted in Biosolv, a water-miscible emulisifier liquid scintillation cocktail. The total amount of radioactivity was taken as a measure of the amount of food ingested.

*Mutagenesis*

Mutagenesis was carried out as described by Brenner [4] using ethylmethanesulfonate.

## RESULTS AND DISCUSSION

Figure 1 shows the mutant screening procedure used to isolate strains with increased life spans. The strain DH26 is a temperature-sensitive spermatogenesis-defective mutant that is sterile at restrictive temperature (25°C), thereby eliminating the complicating presence of progeny during the screening process. DH26 has an average life span indistinguishable from that of the wild type at both 16°C and 25°C [12]. The mutagenized parents were allowed to reproduce by self-fertilization for two generations to allow for homozygosity of recessive mutations. Two generations after the establishment of 1000 F2 "master clones" at 16°C, 1000

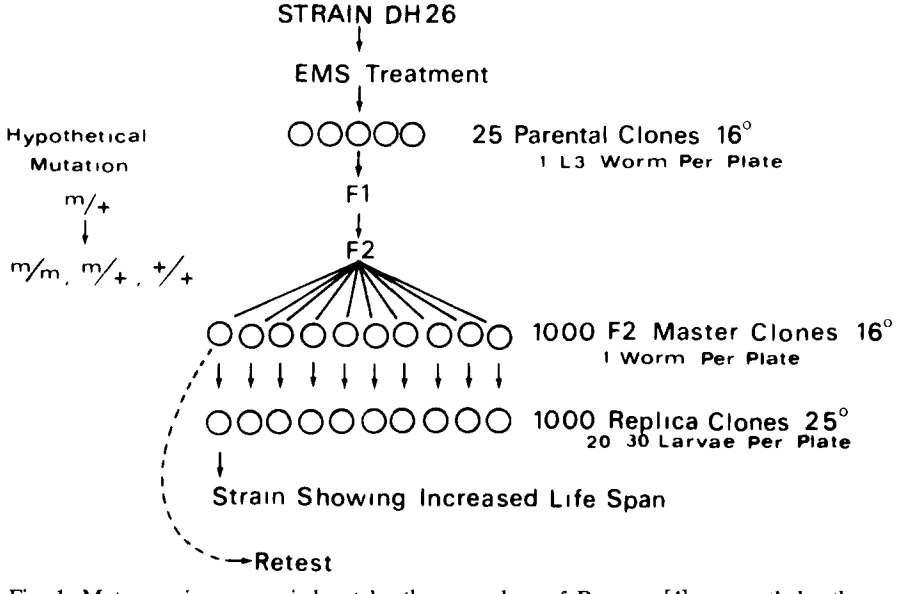


Fig. 1 Mutagenesis was carried out by the procedure of Brenner [4] using ethylmethanesulfonate (EMS). L3 stage larvae were transferred to NGM agar plates seeded with *E coli* strain OP50 [3] after EMS treatment. These "parental clones" were allowed to undergo self-fertilization for two generations at permissive temperature (16°C), after which 1000 F2 progeny were picked and individually transferred to fresh NGM plates (one worm per plate) at 16°C. These "master clones" were allowed to reproduce for several generations after which samples of 20–30 first stage larvae were removed from each clone and transferred to individual plates seeded with a lawn of *E coli* at restrictive temperature (25°C) These "replica clones" were allowed to mature, age, and die. During this process the replica clones were checked at 10, 20, 30, 40, 60 and 70 days after transfer to 25°C to determine the presence of live worms. The mean life span at 25°C is $8.9 \pm 1$ days and the maximum life span is 12 days for the wild type [12]. Those clones having live worms after 20 days were retested by retrieving the master clone and measuring the life span at 25°C Because of the increase in life span caused by dietary restriction [12] care must be taken to insure that the worms on the replica clones have an adequate food supply

"replica clones" were made by transferring 20–30 first-stage larvae to fresh NGM plates with a lawn of *E. coli*. These replica clones were allowed to mature, age and die at 25°C. Any clones exhibiting long-lived worms, *i.e.* worms still alive 20–30 days after transfer, were retested by retrieving the master clone. The results of screening 8000 F2 clones are presented in Table I This screening procedure allowed detection of life span increases as small as 20% over the wild type.

Eight mutants were identified as having significantly increased life spans. Two of the mutant strains spontaneously entered the dauer larva state. This semiquiescent state is characterized by cessation of growth, development and feeding, while maintaining active mobility and chemotactic response [17]. Worms entering the dauer state have significantly increased life spans as previously described [13]. Revertants of the DH26 temperature-sensitive sterile phenotype were also encountered. Both the spontaneous dauer formers and the revertants were discarded.

Of the six remaining mutants, five showed partial paralysis affecting the bilobed muscular pharynx used by the worm to ingest bacteria. All of these mutants demonstrated slightly slower developmental rates. When measured at different ages the pharyngeal pumping rates and rate of food ingestion were always significantly lower than the wild type. Reduced pharyngeal pumping caused a reduction in food ingestion as shown in Table I and Fig. 2. Both reduced

TABLE I

RESULTS OF SCREENING 8000 F2 CLONES

The clone number refers to the number (1–1000) assigned during a particular mutagenesis involving 1000 F2 clones The dauer state has been described previously [13,17]. The mean life span at 25°C is shown with the 95% confidence interval of the mean ($n = 50$) The mean pumping rate was measured on individual worms, viewed through a dissecting microscope, as beats/min and represents the average of 20 different individuals ± the 95% confidence interval of the mean. Food intake was measured by feeding worms (six days' old) $^{35}$S-labeled *E coli* for 2 h, washing and counting total amount of label taken up Data are shown as cpm per worm ± 95% confidence interval of the mean for $n = 12$ worms

| *Mutant clone* | *Mean life span (days)* | *Phenotype* | *Mean pharyngeal pumping rate (beats/min)* | *Mean food intake (cpm/worm)* |
|---|---|---|---|---|
| 782 | >45 days | Spontaneous dauer former | – | – |
| 895 | >45 days | Spontaneous dauer former | – | – |
| 542 | 15.05 ± 1 75 | | 129 ± 25 | 900 ± 20 |
| 546 | 14 05 ± 1.55 | Partial paralysis | 143 ± 20 | 1100 ± 23 |
| 248 | 11 50 ± 0.90 | (reduced pharyngeal pumping rate) | 176 ± 21 | 1273 ± 17 |
| 31 | 11 50 ± 1.05 | | 191 ± 18 | 1260 ± 21 |
| 7 | 11 50 ± 0.95 | | 192 ± 15 | 1276 ± 32 |
| 508 | 15 60 ± 1.50 | Chemotaxis defect | – | – |
| N2 | 9.40 ± 0.50 | Wild type | 220 ± 15 | 2000 ± 31 |

pharyngeal pumping and reduced food ingestion were highly correlated with the increased life spans of the different mutant strains (Figs. 3 and 4). There was a positive correlation of 0.974 between the percentage reduction in pumping rate and the percentage increase in life span as shown in Fig. 3. Likewise there was a correlation of 0.971 between the percentage decrease in food ingestion and the percentage increase in life span (Fig. 4). The increased life spans of these mutants may therefore be due to the reduction in food intake previously shown to lead to an increase in life span [12].

The remaining mutant was observed to have a chemotactic defect, as evidenced by its lack of attraction to the bacterial food source. In fact, when transferred to a fresh NGM culture plate with a small lawn of *E. coli* in the center of the plate and checked periodically, 60–70% of the worms were found outside the bacterial lawn, in contrast to the wild type strain in which 95–100% of the worms were found within the bacterial lawn. This behavior leads to a reduction of food ingestion.

The screening procedure allowed detection of mutants showing an increase in life span by as little as 20% over the wild type. All of the mutants with significantly increased life spans isolated from 8000 mutant F2 clones had increased life spans apparently only as a secondary effect. Two of the eight mutants showed increased life spans due to dauer formation. Six of the eight showed increased life spans presumably as the result of reduced food intake, previously shown to lead to increased life span in the nematode [12] as well as in many other species [18]. Out of the 8000 F2 master clones, there were no mutants that specifically altered only life span.

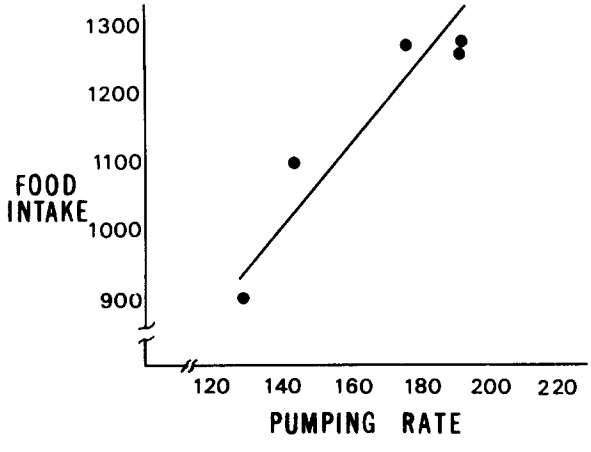


Fig 2 Food intake versus pharyngeal pumping rate. The amount of food ingested was determined as described in the Methods section by feeding worms $^{35}$S-labeled *E coli* for 2 h and counting total radioactivity taken up after washing. Pumping was determined by viewing through a wild dissecting microscope. The percentage reduction in pharyngeal pumping rate was calculated from the data in Table I using 220 as the pumping rate of the wild type. $Y = 5\,35X + 272$ by least-squares method. $r = 0\,937$.

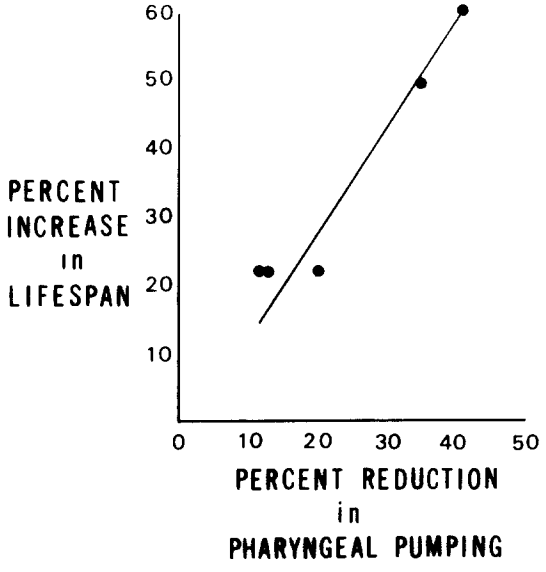


Fig. 3. Percentage increase in life span versus percentage reduction in pharyngeal pumping rate The percentage increase in life span was calculated from the data in Table I using a mean life span of 9.4 days as the wild type The percentage reduction in pharyngeal pumping rate was calculated as in Fig. 2 $r = 0.974$, $Y = 1.36X + 1.85$.

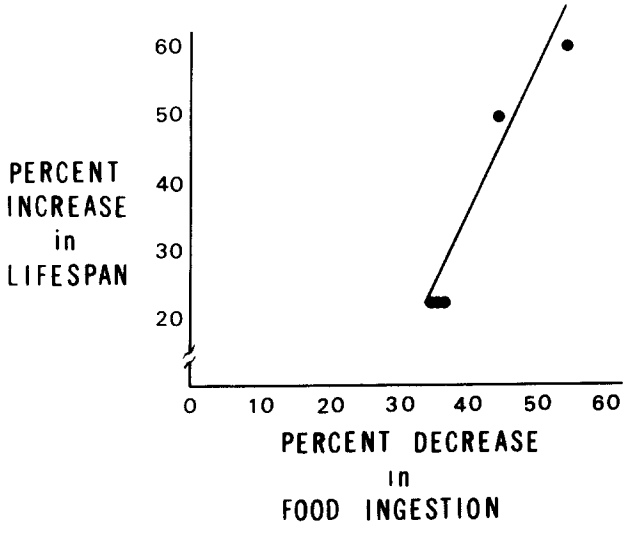


Fig 4 Percentage increase in life span versus percentage decrease in food ingestion. Data are taken from Table I. The percentage decrease in food ingestion was calculated using 2000 cpm as the measure of ingestion for the wild type $r = 0.971$, $Y = 2.14X - 54.6$.

This is an interesting result pertinent to several hypotheses on genetically programmed life span. For example, one hypothesis on the nature of the genetic control on longevity suggests the existence of specific aging genes which are activated at maturity and cause the subsequent senescence of the organism [3].

The results of our screening procedure argue that such genes are rare. There is a small possibility even after backcrossing that the mutations causing paralysis are separate from those causing an increase in life span. Experiments to separate them genetically are now underway (T. Johnson, personal communication). However, it is most probable that none of the mutants with increased life spans bear mutations in specific aging genes. Rather, life span may simply be increased because of the reduction in food uptake caused by paralysis or a chemotactic defect.

A calculation can be made from these results to estimate the number of specific aging genes. Based on the number of lethal mutations we observed that the forward mutation rate in these experiments was very similar to that reported by Brenner ($5 \times 10^{-4}$) [4]. If we use this forward mutation rate and take into account the fact that the vast majority of mutations are recessive, then based on the fact that no mutations were found in specific aging genes in a total of 8000 clones (*i.e.* the frequency of mutations in aging genes was $<1/8000$, or $1.25 \times 10^{-4}$), the number of specific aging genes is equal to

$$\frac{1.25 \times 10^{-4}}{(0.25)(5 \times 10^{-4})}$$

where 0.25 is the probability of homozygosity of a recessive mutation in the F2 generation. This calculation shows that the number of specific aging genes having greater than a 20% effect on life span is less than one. These results therefore suggest that specific aging genes, if they exist at all, must be very rare. Alternatively, life span may be controlled in a polygenic fashion by the interaction of a large number of genes each of which has a very small effect on life span. Our data do not address the question of the cumulative action of multiple sets of independently transmitted genes, each of which produces only a small effect on life span. To answer the question of polygenic control on life span, a different selection scheme must be employed, perhaps one involving the selection of the longest-lived progeny at each generation in the presence of subtoxic levels of a mutagen.

ACKNOWLEDGEMENTS

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