

Caffeine-resistant mutants of *Caenorhabditis elegans*

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Summary

Wild-type *Caenorhabditis elegans* fails to reach adulthood if L1 larvae are incubated in the presence of 30 mM or greater concentrations of caffeine. Eleven mutants have been isolated in which caffeine has a less pronounced effect on development. The mutations are recessive, define two genes, and have been mapped. The mechanism(s) of resistance is unknown.

1. Introduction

The varied effects of the methylxanthine-derivative caffeine on living organisms have received extensive attention, owing in part to the compound's pervasive nature (reviewed by Dews, 1982; Fredholm, 1985; Kihlman, 1977; Timson, 1977). While the majority of such studies have focused upon the effects of sub-millimolar concentrations, which equate with serum levels in humans and model systems, some data have been reported in which higher concentrations were employed. These data indicate that caffeine exerts both direct and indirect effects. In the best-characterized indirect effect, sub-lethal caffeine concentrations act to enhance the lethal actions of a variety of DNA-damaging agents, presumably via the inhibition of DNA repair mechanisms. Yet higher concentrations can induce mutations and chromosome aberrations, perturb a number of biochemical pathways, inhibit cell growth and division and, ultimately, actually kill cells and whole organisms. Unfortunately, because a number of activities have been ascribed to caffeine, it is difficult to clarify the underlying mechanism(s) by which caffeine mediates these events.

One approach towards solving this problem is genetical; that is, isolate and characterize mutants altered in their responses to caffeine. Such mutants, particularly those resistant to the lethal actions of caffeine, have been isolated in a number of organisms (see Discussion for references). In *Escherichia coli* these mutants often display hypersensitivity to DNA-damaging agents (Grigg, 1968; Delvaux & Devoret, 1969). Since our laboratory is engaged in the isolation and characterization of DNA repair-defective mutants in the free-living nematode *Caenorhabditis elegans* (e.g. Hartman & Herman, 1982; Hartman, 1985), we

undertook to isolate caffeine-resistant mutants of *C. elegans*. The isolation of eleven such strains as well as their partial characterization is the subject of this communication.

2. Materials and methods

The methods of Brenner (1974) were employed in stock maintenance and handling. The following genes and alleles were utilized: *him-1(e879)* I, *dpy-5(e61)* I, *unc-13(e1091)* I, *lin-11(n566)* I, *dpy-10(e128)* II, *unc-32(e189)* III, *unc-5(e53)* IV, *dpy-4(e1166)* IV, *dpy-11(e224)* V, *unc-42(e270)* V, *him-5(e1467)* V, and *unc-58(e665)* X. Nomenclature for these, as well as the *caf* mutants, conforms to the system described by Horvitz *et al.* (1979). The wild-type strain was N2.

To isolate the *caf* mutants, N2 hermaphrodites were mutagenized with ethyl methanesulphonate (Brenner, 1974). Synchronous populations of F3 embryos were obtained by dissolving gravid F2 hermaphrodites in alkaline bleach as described previously (Hartman & Herman, 1982). These were plated on NG agar containing 40 mM caffeine and a lawn of *Escherichia coli*. Animals capable of developing to the L3 stage or further were retested by scoring their progeny on 30 mM caffeinated plates as described below. To ensure that each isolate was independent, only one mutant from each mutagenized plate was retained for genetic analyses.

Animals were typically scored for the Caf phenotype by allowing adults to lay eggs and transferring the L1 larvae to NG agar plus 30 mM caffeine after 24 h. Plates were examined two and three days later. While a majority of caffeine-resistant animals were healthy L4 larvae or adults, caffeine-sensitive animals were typically sickly and staged L2 or younger. This procedure was employed for all genetic analyses. In

several instances, a particular visible mutation significantly reduced the resistance conferred by the *caf* mutation. This was particularly troublesome in determining the linkage of *caf-2*. In these cases, 20 mM caffeine plates were used. Under these conditions, some wild-type animals would attain adulthood, but the only visible mutants to show such development contained the *caf* mutation. This was confirmed by outcrossing and recovering the *caf* mutation in a wild-type background.

The methods of genetic analysis, particularly those unique to *C. elegans*, were as described by Herman & Horvitz (1980). Complementation testing was performed by crossing the mutant hermaphrodites of interest with either *him-5;caf-1(hf3)* or *him-5;caf-2(hf5)* males and examining the progeny for males which were resistant to caffeine. There were no ambiguities in complementation testing. The visible mutations *dpy-5*, *dpy-10*, *unc-32*, *dpy-4*, *dpy-11* and *unc-58* were employed to ascertain linkage, using *caf-1(hf3)* and *caf-2(hf5)*. Three-factor crosses were conducted by crossing the appropriate double mutant (*dpy unc*) by either *him-5;caf-1(hf3)* or *him-5;caf-2(hf5)*, picking individual second-generation recombinants to separate plates (e.g. *dpy caf+ /dpy+unc* or *dpy++ /dpy+unc*), and scoring their progeny for caffeine resistance. In two-factor crosses, a *cis* double heterozygote was constructed, using either *caf-1(hf3)* or *caf-2(hf5)* and the appropriate visible mutation. Complete broods of these heterozygotes were then tested for caffeine resistance. Map distances were calculated from the formula $p = 1 - \sqrt{(1-R)}$, where R was the fraction of caffeine-resistant animals which did not also show the visible phenotype. A *caf-1(hf3);caf-2(hf5)* double mutant was constructed by crossing *caf-2;him-5* males by *caf-1;dpy-5* hermaphrodites. Non-Dpy, caffeine-resistant F2s were tested individually for their abilities to segregate Dpy offspring, the presence of which suggested a *caf-1/caf-1;dpy-5/caf-2* genotype. Non-Dpy F3s were cloned, and presumptive double mutants (suggested by the absence of Dpy progeny) were complementation tested with both *caf-1* and *caf-2* as described above.

Sensitivities to UV radiation were determined as before (Hartman & Herman, 1982). In brief, gravid hermaphrodites were dissolved in alkaline bleach to yield synchronous populations of eggs (young embryos). These were diluted appropriately, plated, and either exposed immediately or incubated for 24 h before irradiation. Survival was scored after 4–5 days as the number of animals which attained adulthood. A single 15 W bulb (G15T8), at a distance of 55 cm, was employed to deliver a fluence rate of 0.5 W m^{-2} as determined by ferrioxalate actinometry (Jagger, 1967).

Caffeine and theophylline were purchased from Sigma (St Louis, MO) and were added to the medium prior to autoclaving. Bacteria were concentrated

20-fold by centrifugation before serving as surface inocula, since greater than 10 mM caffeine or theophylline otherwise reduced lawn thicknesses greatly. To quantitate the effects of theophylline and caffeine on growth and survival, 20 L1s were picked to a series of plates. After three days the plates were placed in a 55 °C incubator for 25 min, killing the animals while not altering their lengths (Byerly, Cassada & Russell, 1976).

Food intake was measured as described by Klass (1983), except that L1 animals were incubated for 2 h on agar plates upon which $20 \mu\text{Ci}$ [^3H]thymidine was spread before bacterial inoculation.

3. Results

If L1 larvae of the wild-type strain (N2) are plated in the presence of 30 mM or greater concentrations of the methylxanthine-derivative caffeine, most animals fail to develop past the L3 stage. Eleven *caf* (for caffeine resistance) mutants were isolated as described in Materials and methods. Under the same experimental conditions a majority of these animals attained adulthood. Each was outcrossed twice to wild type and each behaved as a single, recessive mendelian factor. Complementation testing indicated that the mutations define two genes, named *caf-1* and *caf-2*.

There are eight alleles of *caf-1* (*hf3*, *hf4*, *hf6–hf9*, *hf11–hf14*) and three alleles of *caf-2* (*hf5*, *hf10*, *hf15*). After assignment to LGIV, a three-factor cross using *unc-5* and *dpy-4* indicated *caf-1* to be to the left of *unc-5*. Two-factor crosses placed *caf-1* 15 mu away from *dpy-4* (35 out of 130 caffeine-resistant animals were non-Dpy) and 5 mu away from *unc-5* (22 out of 228 caffeine-resistant animals were non-Unc). These data differ in their positioning of *caf-1* by less than one map unit. After a three-factor cross involving *unc-13* and *dpy-5* placed *caf-2* to the right of *unc-13*, it was shown to be located approximately 7 mu away from *dpy-5* (21 out of 190 caffeine-resistant animals were non-Dpy). These data are in agreement with the observation that *nDf24/caf-2* animals were resistant to caffeine. The *caf-2* mutation was placed opposite this deficiency by crossing *caf-2* males with *nDf24/unc-13 lin-11* hermaphrodites. Caffeine-resistant progeny were self-crossed and segregated approximately one-fourth inviable zygotes (presumed *nDf24* homozygotes), but no Unc or Lin animal.

Both *caf-1* and *caf-2* were tested to see if they influenced spontaneous mutability. This was accomplished by examining for non-Unc revertants in an *unc-58* background (Hodgkin, Horvitz & Brenner, 1979). Such revertants arise by mutations at the *unc-65* locus or close to it (Hodgkin *et al.* 1979). Approximately 30 hermaphrodites of either *caf-1* or *caf-2* were placed on each of 20 15×100 mm petri dishes and grown to starvation. There was no revertant on any of the 40 plates, with approximately 10^5 animals per plate at starvation. Since this assay has been

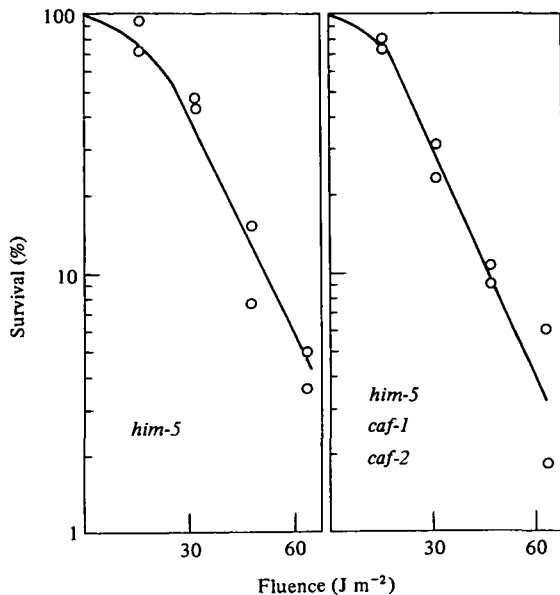


Fig. 1. UV-radiation sensitivities of *him-5* and *him-5; caf-1; caf-2* embryos.

successfully employed to demonstrate increased levels of spontaneous mutation (e.g. 31 out of 34 such plates contained revertants after inoculation with *rad-5; unc-58* – Hartman & Herman, 1979), it is concluded that the *caf* genes do not dramatically influence spontaneous mutability.

The sensitivities to two DNA-damaging agents, UV radiation and methyl methanesulphonate, were also tested. Embryos which were either wild type or doubly mutant for *caf-1* and *caf-2* were exposed to graded effluences of 254 nm radiation, and survival was scored as the percentage of animals capable of developing into adults (Fig. 1). The results were experimentally identical, as evidenced by F_{37} values, where F_{37} is the fluence required to reduce survival to 37% that of unirradiated controls. The values for the two strains were 32 and 29. These are similar to the value of 33 reported several years ago for wild type (Hartman, 1984). The two strains also displayed similar sensi-

tivities if irradiated as L1s, or if exposed to MMS (data not shown).

The effects of the *caf* mutations on genetic recombination were measured (Table 1). When recombination was measured over the *dpy-11 unc-42* interval on linkage group V, the *hf3* allele was shown to elevate recombination frequency slightly. However, the *hf4* allele of *caf-1*, as well as *caf-2*, had no effect on recombination, as measured across this interval. Moreover, the *hf3* allele of *caf-1* did not affect the rate of crossover between *dpy-5* and *unc-13* on linkage group I. Therefore, the elevated recombination frequency observed with the *hf3* allele is probably due to a second mutation in the genetic background. Although more exhaustive experimentation could have been conducted, these results suggest that mutations in the *caf* genes do not profoundly influence the frequencies of genetic recombination.

The effects of caffeine on the growth of L1s were quantitated by measuring animals' lengths after three days incubation on caffeine-containing plates (Fig. 2; data not shown). At concentrations between 20 and 40 mM, the *caf* mutants were roughly one and one-half times longer than wild type. In addition, at 30 mM many *caf* mutants attained adulthood, as evidenced by egg laying; whereas wild-type animals rarely developed to this extent. In fact, concentrations of 30 mM and greater resulted in significantly higher mortality in wild types than in the *caf* mutants. All alleles were indistinguishable in their abilities to confer caffeine resistance. The *caf-1; caf-2* mutant was more resistant than either of the single mutants (Fig. 2).

Wild type and the *caf* mutants were similarly tested with the closely related methylxanthine-derivative theophylline (Fig. 3). Equivalent concentrations of theophylline had comparatively little effect on length. It appears that the *caf* mutations may also confer resistance to theophylline, although the relatively poor solubility of theophylline, coupled with the extreme resistance of wild type, made it impossible to determine this rigorously.

A reduced rate of food intake could confer caffeine

Table 1. Recombination frequencies in the *caf* mutants over selected intervals

<i>caf</i> genotype	Segregation from <i>dpy-11 unc-42 V/+ +</i>		Segregation from <i>dpy-5 unc-13 I/+ +</i>	
	Percentage linkage*	χ^2 relative to wild type	Percentage linkage*	χ^2 relative to wild type
Wild type	3.2 (1894)	—		
<i>caf-1(hf3)/+</i>	3.0 (1738)	0.3	4.3 (670)	—
<i>caf-1(hf3)/caf-1(hf3)</i>	4.6 (1611)	8.8	3.8 (629)	0.3
<i>caf-1(hf4)/caf-1(hf4)</i>	3.3 (1239)	0.2		
<i>caf-2(hf5)/+</i>	3.6 (1400)	0.3		
<i>caf-2(hf5)/caf-2(hf5)</i>	3.4 (2062)	0.2		

* Numbers in parentheses indicate total number of animals examined.

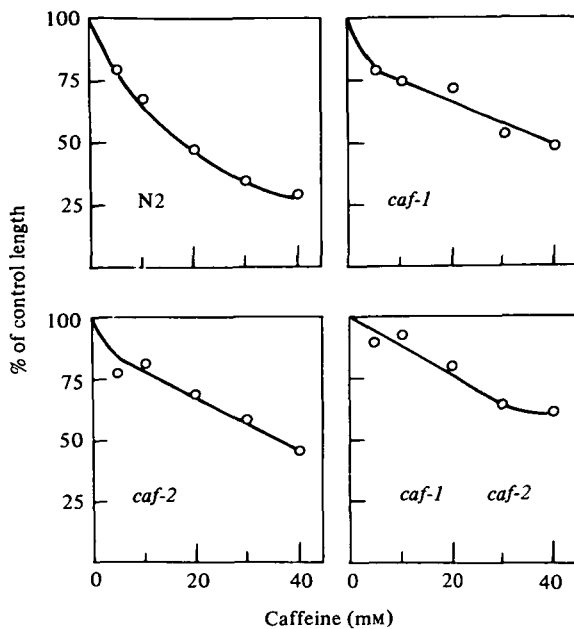


Fig. 2. Effects of caffeine on growth. L1 larvae were incubated on caffeinated petri plates for 48 h and their lengths measured as described in Materials and methods.

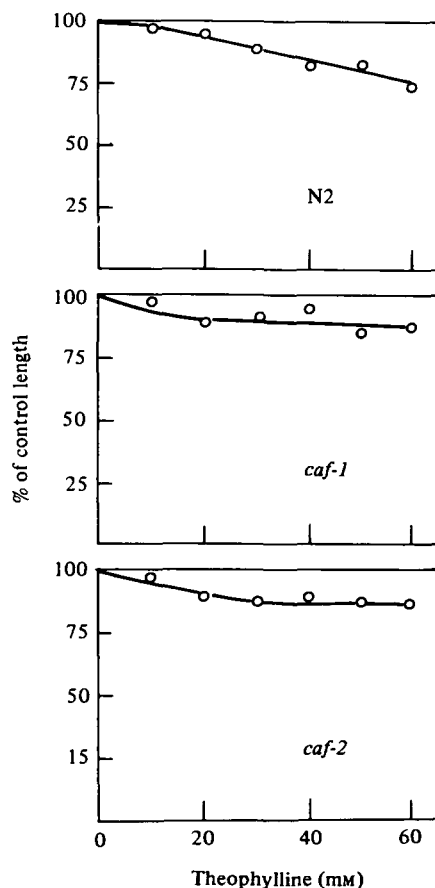


Fig. 3. Effects of theophylline on growth. L1 larvae were incubated on petri plates containing theophylline for 48 h and their lengths measured as described in materials and methods.

resistance if it allowed the nematode longer to metabolize caffeine in the lumen of the gut. This was tested by feeding caffeine-resistant (*him-5; caf-1; caf-2*) and caffeine-sensitive (*him-5*) animals radioactively labelled bacteria for 2 h, washing and counting radioactive uptake. The experiment was performed twice, with duplicate plates for each strain. The mean food intake (measured as cpm/worm) of the caffeine-resistant animals was 94% and 104% of the caffeine-sensitive animals for the two experiments. Similar experiments were performed using plates containing 30 mM caffeine. As before, the mean food intake was substantially the same for two strains; specifically, the intake of the caffeine-resistant animals was 87% and 108% that of the caffeine-sensitive animals.

4. Discussion

This communication reports the isolation and characterization of mutants of the nematode *C. elegans* which are resistant to caffeine. These represent, to our knowledge, the first example of such mutants in a multicellular organism. However, caffeine-resistant mutants have been isolated in other organisms, including *E. coli* (Grigg, 1968; Delvaux & Devoret, 1969), *Candida albicans* (Sarachek, Bish & Ireland, 1970), *Penicillium* (Woolfolk, 1975), and *Schizophyllum commune* (Klein & Deppe, 1985). The existence of such mutants raises two interrelated questions. First, what are the underlying mechanisms by which caffeine exerts its lethal effects? Secondly, how are the mutants altered to minimize the toxic effects of caffeine?

There are at least five explanations for caffeine resistance in the *C. elegans* *caf* mutants. We present evidence that argues against three of these. It was initially hoped that the mutants were altered in their DNA repair capacities. This proved to be the case with many caffeine-resistant mutants of *E. coli*, as evidenced by their altered sensitivities to DNA-damaging agents (Grigg, 1968; Delvaux & Devoret, 1969). The observations that, in some eukaryotes, caffeine alone can induce DNA damage (Ishida, Kozaki & Takahashi, 1985) as well as inhibit some forms of DNA repair (reviewed by Kihlman, 1977) made it logical to assume that a similar explanation could hold for *C. elegans*. However, while DNA repair levels were not measured directly in the caffeine-resistant mutants of *C. elegans*, the normal sensitivities to UV radiation and MMS as well as unaltered recombination and spontaneous mutation frequencies argue against such an involvement. In this context it deserves mention that caffeine does not inhibit DNA repair in *C. elegans* (Keller *et al.* 1986).

As a second explanation for the mechanism of caffeine resistance, it could be supposed that caffeine inhibits cell division in the nematode. While caffeine-induced perturbations in mitosis and cell division are

well documented (e.g. Kihlman, 1977; Beetham & Tolmach, 1982), and while caffeine may inhibit cell division in *C. elegans*, the developmental biology of this organism indicates this hypothesis to be untenable. Specifically, the increase in size throughout development is due largely to increase in cell size rather than cell number. In fact, many cells in this animal undergo no post-embryonic cell division (Sulston *et al.* 1983). Indeed, there are many mutations which act to alter cell lineages, including those in the *lin-5* gene, which block virtually all post-embryonic cell divisions (Sulston & Horvitz, 1981). Despite this, *lin-5* hermaphrodites grow normally and reach adulthood. By analogy, if the major effect of caffeine on wild type was to inhibit cell divisions, these animals should develop into adults in the presence of caffeine, albeit they should be sterile. By the same token, the possibility that caffeine inhibits growth by perturbing cell divisions appears unlikely, since there are other lineage mutants with aberrant patterns of cell division, yet they develop into full-sized adults (e.g. Sulston & Horvitz, 1981).

A third explanation for caffeine resistance is that the *caf* mutants have a reduced rate of pharyngeal pumping, thus ingesting caffeine at a slower rate and allowing a greater time for its metabolism into non-toxic by-products. Mutations that act to reduce food intake can result in pleiotropy, as evidenced by the fact that some were isolated in screens for long-lived mutants (Klass, 1983). This appears not to be the case with the *caf* mutants, as the double mutant has the same rate of food uptake as wild type. However, these data do not address the possibility of a gut caffeine uptake defect in the resistant mutants.

It also seems possible that the *caf* mutants are resistant because they more efficiently metabolize caffeine. Crude attempts to measure caffeine metabolism in *C. elegans* were unsuccessful; however, more sophisticated methodology, such as HPLC, could resolve this possibility.

A related, alternative explanation for caffeine resistance is that the mutants are altered in some metabolic function which is sensitive to caffeine. Caffeine affects a wide variety of metabolic functions (Kihlman, 1977; Timson, 1977; Dews, 1982). In particular, caffeine effects have often been attributed to its inhibitory action on phosphodiesterase, consequently elevating cAMP levels. However, this notion is open to question (Fredholm, 1985). Moreover, if true for *C. elegans*, animals would be expected to be more sensitive to theophylline than caffeine, since theophylline has been shown to be a more potent inhibitor of phosphodiesterase in a number of other organisms. Since *C. elegans* was less sensitive to theophylline than caffeine, it is tempting to speculate that the mechanism of caffeine resistance does not involve phosphodiesterase. However, this interpretation may be confounded by differential uptake or metabolism rates of

caffeine and theophylline. Substantive data are clearly lacking to make any definitive statement regarding the role of the *caf* gene products in metabolism.

As mentioned previously, more extensive investigations of the *caf* mutants may not only reveal the mechanism of caffeine resistance, but perhaps more importantly indicate how caffeine kills animals. However, two important limitations need to be mentioned. First, the mutants are only partially resistant to caffeine. While resistance is a stable and reproducible phenotype, the cellular component(s) may be so subtly affected by the mutations as to escape biochemical notice. Secondly, all experiments were performed with animals grown on *E. coli* as a food source. Thus the possibility exists that the mutants are resistant not to caffeine, but to a product metabolized by the bacteria. These reservations notwithstanding, it is hoped that these mutants will be of assistance in understanding mechanisms of caffeine toxicity.

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