Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 819–823, February 1984
Genetics

A pheromone-induced developmental switch in *Caenorhabditis elegans*: Temperature-sensitive mutants reveal a wild-type temperature-dependent process

(nematode/dauer larva/informational suppression)

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Communicated by William B. Wood, September 12, 1983

ABSTRACT **Formation of a developmentally arrested dispersal stage called the dauer larva is enhanced by a *Caenorhabditis*-specific pheromone and is inhibited by increasing amounts of food. Pheromone-induced dauer larva formation of three tested wild-type strains is temperature-dependent, so that an increased percentage of the population forms dauer larvae at 25°C compared to lower temperatures. Dauer-defective mutants fail to respond to added pheromone, and some behavioral mutants affected in thermotaxis or egg-laying also exhibit abnormal responses. Temperature-sensitive (*ts*) dauer-constitutive mutants form dauer larvae at a restrictive temperature regardless of environmental stimuli. At the permissive temperature (17.5°C), alleles of six out of seven dauer-constitutive genes tested overrespond to the dauer-inducing pheromone. All known mutations in *daf-4* (eight alleles) and *daf-7* (five alleles) produce a *ts* dauer-constitutive phenotype. One *daf-4* and one *daf-7* allele are suppressed by the amber nonsense suppressor, *sup-7(st5)*. At least these two dauer-constitutive mutations are likely to cause production of nonfunctional rather than *ts* gene products. These mutations appear to indirectly result in a *ts* phenotype by enhancing the expression of a wild-type *ts* developmental process.**

Temperature-sensitive (*ts*) mutations allow the propagation of strains containing lesions in essential genes. Furthermore, the temperature-sensitive period (TSP) in development can be determined by appropriate temperature-shift and temperature-pulse experiments (1). The TSPs or execution stages of many *ts* mutants have been determined as an indication of the developmental time of gene product synthesis or action (2). The interpretation of data on TSPs is often complicated by the complexity of the developmental processes being studied, particularly in cases where the gene product has not been identified. Such is the case for *ts* mutants of the soil nematode, *Caenorhabditis elegans*, which have been used in the study of embryogenesis, gonadogenesis, sex determination, fertilization, and dauer larva formation (3–8).

The *C. elegans* dauer larva is a facultative, developmentally arrested, nonfeeding dispersal stage that differs from all other stages in both behavior and morphology (9, 10). Dauer larvae are induced to form at the second larval molt by an appropriate combination of environmental stimuli, which include a dauer-inducing pheromone, the food supply, and, as we show here, temperature. The concentration of the *Caenorhabditis*-specific pheromone apparently serves as a measure of population density. The pheromone is a stable, nonvolatile, fatty acid-like compound or family of compounds (11). The conditions that favor dauer larva formation also inhibit exit from the dauer stage; but when dauer larvae are transferred to a recovery-inducing environment, with an appropriately high ratio of food to pheromone, they begin to feed and then molt to resume development (9, 11, 12).

We have studied the genetics of dauer larva formation with the goal of understanding how a subset of the animal's genes specify a simple developmental sequence. Dauer-constitutive mutants of *C. elegans* form dauer larvae independently of the environmental cues and fail to reach reproductive maturity (8). However, *ts* constitutive mutants form dauer larvae at high frequency only at restrictive temperatures, and if such larvae are shifted to permissive temperatures, they exit from the dauer stage and resume growth. A second mutant type, called dauer-defective, is unable to form dauer larvae (13, 14). A genetic pathway for dauer larva formation has been constructed by analyzing epistatic relationships between dauer-defective and *ts* dauer-constitutive mutants (15). Execution stages for six *ts* dauer-constitutive mutations have been defined (8).

We show here that in wild-type populations, a high ratio of pheromone to food increases the frequency of dauer larva formation in a temperature-dependent manner, and dauer-constitutive mutants reveal the natural temperature-dependence.

MATERIALS AND METHODS

Strains and General Culture Methods. Strains obtained from the *Caenorhabditis* Genetics Center include the thermotaxis-defective strains PR761, PR767, and PR771, previously designated EH61, EH67, and EH71, respectively (16). The three egg-laying defective mutants, MT1236 (*egl-40*), MT155 (*egl-32*), and MT1073 (*egl-4*) were from Carol Trent. All mutant *C. elegans* strains are derivatives of the wild-type Bristol strain N2, and those originating in our laboratory were backcrossed with N2 males prior to further analysis. Genetic nomenclature has been described (17). The wild-type *C. briggsae* strain G16 was brought to our laboratory by A. Fodor. Unless otherwise indicated, worms were grown on NG agar plates streaked with *Escherichia coli* strain OP50 (18) in forced-air incubators that maintain temperature to ±0.5°C.

Pheromone Extract. The stability of the fatty acid-like pheromone permitted the use of the following procedures, which provided >90% recovery of biological activity. One liter of exhausted liquid culture medium (11) was reduced in volume by drying under a stream of air at 100°C and then centrifuged at 10,000 × *g* for 10 min. The supernatant was completely dried in a vacuum oven at 60°C. This residue was extracted 4–6 times with 95% ethanol until the extract was

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Abbreviations: ts, temperature-sensitive; TSP, temperature-sensitive period.

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only slightly colored. The extracts were combined and dried under a stream of air at 60°C. The resulting oily residue was then back-extracted into 10 ml of distilled water, filtered through Whatman 3-mm paper, sterilized by autoclaving, and stored at 2°C. Each batch of pheromone extract was assayed for activity to determine the concentration that induced ≈75% N2 dauer larvae at 25.4°C. Typically, 20–50 μl of pheromone stock solution was needed per 2-ml agar plate.

Induction of Dauer Larva Formation on Pheromone Plates. Indicated amounts of pheromone extract were added to NG agar prepared without peptone, and 2-ml aliquots were placed into 35 × 10 mm Petri plates. After the agar solidified, 20 μl of a 5% (wt/wt) suspension of *E. coli* strain OP50 in S medium (19) containing streptomycin (2.5 mg/ml) were spotted onto the agar surface. Inhibition of bacterial growth on the plate minimizes the production of "food-signal" (11) and, hence, enhances the effect of the pheromone. The plates then were allowed to dry for 12 hr. Ten gravid adult worms were placed onto each pheromone plate, allowed to lay 80–120 eggs, and then removed from the plates by aspiration. The plates were incubated at the desired temperature until the worms that did not form dauer larvae had become adults. Plates were scored visually by using a Wild M5 stereomicroscope to determine the percentage of the population that formed dauer larvae.

Temperature-Dependence of Dauer Larva Formation. For testing the wild-type nematode strains, pheromone plates were prepared as described above containing 25 μl of pheromone extract per 2 ml of medium and incubated at the indicated temperatures. The two dauer-constitutive mutants DR62 and DR72 were tested on standard NG agar plates started with ≈100 eggs; the plates were incubated at the appropriate temperature for 3–5 days and scored for percentage of dauer larvae.

Suppression of *ts* Dauer-Constitutive Mutants. Suppression tests were performed by the method of Waterston (20) at 22.5°C because *sup-7* strains are only fertile when grown between 22 and 24°C. Briefly, F1 males issuing from a cross between DR478 [*unc-13(e450)I*; *sup-7(st5)X*] and N2 males were mated with dauer-constitutive mutants, and phenotypically wild-type cross-progeny were cloned. From the self-progeny of the triply heterozygous hermaphrodites, partially suppressed (*sup-7*/+) Unc hermaphrodites were sub-cloned, and their progeny that formed dauer larvae were scored for their Unc phenotype. The segregation of suppressed-Unc dauer larvae indicated that the *daf* allele was not suppressed, whereas the segregation of only Unc dauer larvae indicated suppression of the *daf* mutation. Homozygous *unc-13*; *daf*; *sup-7* triple mutants were crossed with N2 males, and heterozygous F1 progeny were cloned to confirm that they segregated all three alleles. To determine whether suppression was dominant or recessive, *daf*/+ males were crossed with *unc-13*; *daf*; *sup-7* hermaphrodites. The absence of F1 dauer larvae showed that suppression was dominant.

RESULTS

Wild-Type Dauer Larva Formation Is Temperature-Dependent. By addition of partially purified pheromone to the agar medium, wild-type strains can be induced to form dauer larvae when they are on a lawn of *E. coli* that is more than sufficient to support growth and reproduction. Pheromone obtained from *C. elegans* strain N2 can induce dauer larva formation in wild-type strains of both *C. elegans* and *C. briggsae*. Incubation of identical plates at various temperatures between 15 and 25°C showed that an increased percentage of the population was induced to form dauer larvae at temperatures above 20°C for the three wild-type strains tested: *C. elegans* strains N2 and DH424 and *C. briggsae* strain G16 (Fig. 1). Data could not be obtained for the strain G16 at 15°C because it grows poorly at this temperature. The temperature-dependence of strain N2 in the presence of added pheromone was similar to that exhibited by many of the *ts* dauer-constitutive mutants in the absence of exogenous pheromone (8), showing a transition in the response between 20 and 25°C. The *ts* dauer-constitutive mutants were further analyzed to determine if their *ts* phenotype might represent the abnormal expression of the wild-type temperature-dependence.

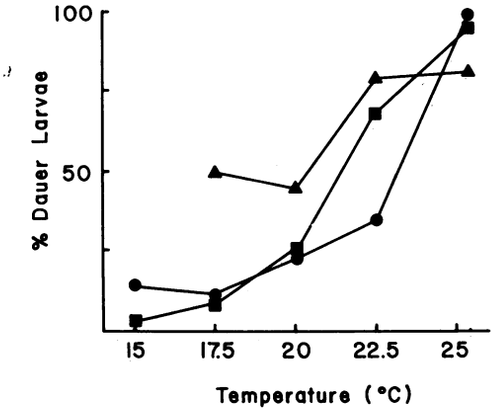

FIG. 1. Temperature-dependence of dauer larva formation for the wild-type *C. elegans* strains N2 (●) and DH424 (■) and *C. briggsae* strain G16 (▲). For each strain, pheromone-containing plates with ≈100 eggs were incubated at the indicated temperatures for the length of time necessary for the growing worms to reach the adult stage, and the percentage of the population that had formed dauer larvae was scored.

Response of Wild-Type and Mutant Nematodes to the Dauer-Inducing Pheromone. The fraction of a synchronously growing population that arrests development at the dauer stage depends on the amount of pheromone added to the medium. In contrast to wild-type strains, most *ts* dauer-constitutive mutants produce 100% dauer larvae at 25°C when grown on *E. coli* without added pheromone and 0–10% dauer larvae at 17.5°C, their permissive temperature (8). Exceptions are *daf-11(m47)* and *daf-4(m72)*, which formed dauer larvae at 17.5°C at frequencies of 15% and 40%, respectively. Fig. 2 shows the pheromone-induced response of the wild-type strain N2 and nine *ts* dauer-constitutive mutants (representing seven genes) as measured at 17.5°C. At this temperature, strain N2 was induced to form only about 30% dauer larvae at the highest concentration of pheromone used. All but two of the tested mutant strains greatly overresponded to pheromone in comparison with N2. This overresponse shows that the mutants behave abnormally even at their "permissive" temperature. The *daf-8(e1393)* strain only slightly overresponded, and the *daf-2(e1370)* strain underresponded. The dose-dependent response to pheromone varied between the different mutants and even between different alleles of the same gene (*daf-4* alleles *m63* and *m72* and *daf-7* alleles *e1372* and *m62*), indicating that allelic differences result in quantitative differences in phenotype. The dose-dependent responses of the strains shown in Fig. 2 were determined by using a single lot of pheromone extract to insure that all strain comparisons were internally consistent. An N2 control, included with each mutant test, was quite reproducible, so that standard errors for each N2 data point were less than ±5 (Fig. 2).

Strain DH424, a recently isolated wild-type strain (21) that is interfertile with N2, was compared with N2 in an experiment identical to those described in Fig. 2, except that a different pheromone preparation was used. The two different wild-type isolates of *C. elegans* showed virtually identical dose-dependent responses to the pheromone.

In contrast to the typical dauer-constitutive mutant, dauer-defective strains were not induced to form any dauer

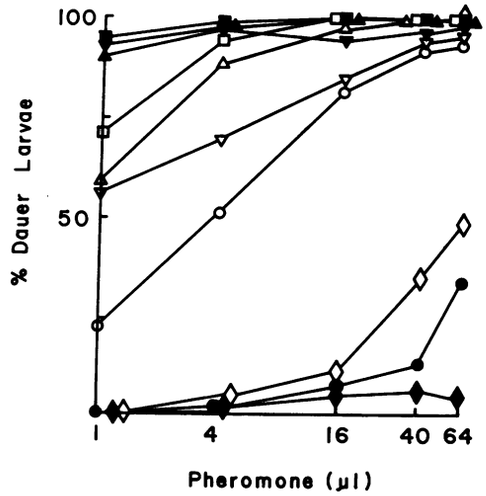

FIG. 2. Sensitivity of dauer-constitutive mutants to the dauer-inducing pheromone. Duplicate or triplicate plates for each data point were prepared with the indicated amounts of pheromone extract per 2-ml plate. Worms hatched from eggs were incubated at 17.5°C for 3 days, and the plates were scored for the percentage of the population that had formed dauer larvae. The strains tested were: wild-type strain N2 (●); DR40, *daf-1(m40)* (△); DR47, *daf-11(m47)* (▲); DR62, *daf-7(m62)* (▽); DR63, *daf-4(m63)* (■); DR72, *daf-4(m72)* (□); DR77, *daf-14(m77)* (○); CB1370, *daf-2(e1370)* (◆); CB1372, *daf-7(e1372)* (▼); CB1393, *daf-8(e1393)* (◇). Standard errors (not shown) for all data points averaged ±4.7.

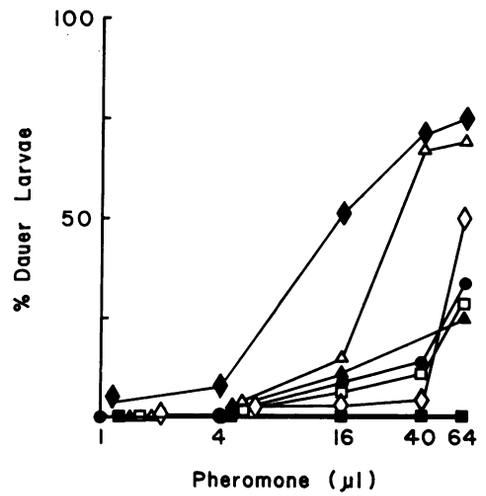

FIG. 3. Sensitivity of behavioral mutants to the dauer-inducing pheromone. Same as Fig. 2 except that the following strains were tested: MT155, *egl-32(n155)* (□); MT1073, *egl-4(n478)* (△); MT1236, *egl-40(n606)* (◇); PR761 (■); PR767 (◆); PR771 (▲). Data for strain N2 (●) are included for comparison. The PR strains carry complementing, recessive thermotaxis-defective mutations that have not been assigned gene designations (16).

larvae at 25°C under conditions that induced 90–100% dauer larvae in an N2 population. Dauer-defective mutants representing 10 genes were all unresponsive to the pheromone on plates containing 64 μl of pheromone extract per 2 ml of medium. These were *daf-3(e1376)X*, *daf-5(e1386)II*, *daf-6(e1377)X*, *daf-10(e1387)IV*, *daf-12(m20)X*, *daf-16(m26)I*, *daf-17(m27)I*, *daf-18(e1375)IV*, *daf-20(m25)X*, and *che-3(e1378)I*.

Some behavioral mutants affected in egg-laying (22) or thermotaxis (16) are abnormal in processing environmental stimuli that also affect dauer larva formation. The egg-laying (*egl*) mutants apparently fail to respond normally to food, and thermotaxis-defective mutants are unable to move along isothermal lines in a temperature gradient. These mutants were not originally characterized as being affected in dauer larva formation but were found to respond abnormally to pheromone (Fig. 3). Unlike the *daf* mutants, however, sensitivity to pheromone could not be predicted by the mutant's primary phenotype. Of three egg-laying defective mutants tested, MT155 [*egl-32(n155)*] was nearly wild-type, MT1073 [*egl-4(n478)*] was overresponsive, and MT1236 [*egl-40(n606)*] was overresponsive at 17.5°C and even more so at 25°C (data not shown). Three tested thermotaxis-defective strains produced three different responses: PR761 failed to produce any dauer larvae under the conditions used (although it produced some dauer larvae on starved plates), PR771 showed a wild-type response, and PR767 overresponded.

Two *ts* Dauer-Constitutive Mutants Carry Nonsense Alleles. Strains were constructed that contain both a dauer-constitutive mutation and the amber nonsense suppressor *sup-7(st5)X* (20, 23, 24). Of 16 *ts* dauer-constitutive alleles tested, two alleles, *daf-4(m72)* and *daf-7(m62)*, were suppressed by *sup-7* (Table 1). The dauer-constitutive phenotype of both alleles was dominantly suppressed, indicating that the gene products are used catalytically rather than stoichiometrically (25).

The *daf-4* and *daf-7* alleles have other unusual properties for *ts* mutations. All mutations in *daf-4* and *daf-7* (listed in Table 1) produce a *ts* dauer-constitutive phenotype, and all alleles of each gene also have a secondary phenotype expressed even at permissive temperature. The *daf-4* mutants are small and both *daf-4* and *daf-7* adults are egg-laying deficient (22). These secondary phenotypes were also suppressed by *sup-7*.

Non-*ts* dauer-constitutive mutations may be rare. In an attempt to bias a dauer-constitutive mutant selection (15) against *ts* alleles, a selection was performed at 15°C after ethyl methanesulfonate-mutagenesis. Five mutants were detected among the progeny of 9,200 F1 animals. All five mutants were *ts*, some of which formed constitutive dauer larvae only rarely at 15°C.

Temperature-Dependent Dauer Larva Formation of DR62 and DR72. Fig. 4 shows the ts phenotype of the two nonsense mutants DR62 [*daf-7(m62)*] and DR72 [*daf-4(m72)*] in the absence of exogenous pheromone. Although both strains produce 100% dauer larvae at 25°C, their responses differ at other temperatures. This difference may reflect the greater sensitivity of *daf-4* mutants to pheromone (Fig. 2). Because the dauer-constitutive mutants differ from N2 and from each other in overall sensitivity to the pheromone, it is not possible to directly compare the degree of temperature-dependence of the *ts* dauer-constitutive mutants on standard NG agar (Fig. 4) with that of N2 in dauer-inducing conditions (Fig. 1).

DISCUSSION

The wild-type response to the dauer-inducing pheromone is temperature-dependent. The influence of temperature appears to provide an adaptive function because it has been conserved within the genus *Caenorhabditis*; wild-type *C.*

Table 1. Suppression of *ts* dauer-constitutive mutants by *sup-7*

	Alleles	
Gene	Suppressed	Not suppressed
daf-1	—	*m40*
daf-2	—	*e1370*
daf-4	*m72*	*e1364, m44, m46, m63, m76, m78, b11*
daf-7	*m62*	*e1372, m70, m83, m88*
daf-14	—	*m77*

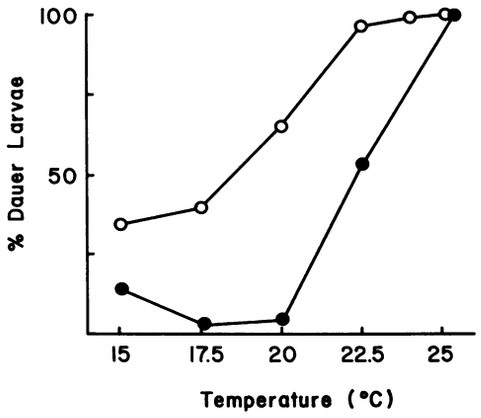

FIG. 4. Temperature-dependence of dauer larva formation for strains DR62, *daf-7(m62)* (●), and DR72, *daf-4(m72)* (○) in the absence of exogenous pheromone. For each strain, NG agar plates containing ≈100 eggs were incubated at the indicated temperatures and scored as in Fig. 1.

elegans strain N2 and two recently isolated wild strains, *C. elegans* strain DH424 and *C. briggsae* strain G16, show temperature-dependent dauer larva formation in response to pheromone partially purified from N2. The three strains show a similar response to temperature even though the isolation of these strains was well separated by time and location. The N2 strain is a subline of the Bristol variety of *C. elegans* originally isolated in the 1950s in England. The DH424 strain was isolated in California in 1982 (21), and *C. briggsae* G16 was isolated in India in 1980 (26).

Mutants affected in thermotaxis are altered in their response to the dauer-inducing pheromone. At least some thermotaxis-defective mutants respond to pheromone in a temperature-dependent manner (unpublished data), in spite of the fact that these mutants do not respond to temperature gradients as do wild-type animals (16). Thus, if temperature is a sensory cue for dauer larva formation, it is probably mediated by a sensory mechanism different from that used in thermotaxis. Alternatively, the temperature-dependence may represent a later step in the behavioral/developmental sequence, such that the initiation of dauer larva morphogenesis has a temperature-dependent component.

Two properties of wild-type pheromone-induced dauer larva formation are similar to those found for many of the *ts* dauer-constitutive mutants. The transition in the temperature-dependence of dauer larva formation for strain N2 is between 20 and 25°C (Fig. 3); and the TSP is at the L1 molt, a full larval molt before dauer larvae are formed (unpublished data). The TSPs, or execution stages, for most *ts* dauer-constitutive mutants (8) roughly correspond to the timing of the *ts* response of wild-type worms grown in the presence of pheromone. These experiments suggest that the initial discrimination between alternative developmental pathways is made around the L1 molt.

All known mutations in the dauer-constitutive genes *daf-4* and *daf-7* produce a *ts* phenotype, even though some mutations appear to be null alleles. Genetic evidence that these *ts* mutants do not produce a *ts* gene product is provided by the suppression of one allele of each gene by *sup-7*. The *sup-7* gene produces a tRNA that mediates suppression of amber (UAG) terminator codons (24). Consequently, we interpret the suppression by *sup-7* to mean that *daf-4* and *daf-7* produce a protein product and that suppressible mutant alleles of these genes produce a truncated polypeptide. Thus, constitutive dauer larva formation may result from a non-*ts* genetic defect that reveals the wild-type *ts* process. Because the gene products are not known for any of the dauer-constitutive genes, it is not possible to confirm a null phenotype for these mutations at the biochemical level. However, it seems unlikely that two independent, suppressible nonsense mutations in two different genes would both result in the production of thermolabile gene products.

The two nonsense mutants overrespond to the dauer-inducing pheromone at the permissive temperature to a lesser degree than do strains carrying nonsuppressible alleles of the same gene (Fig. 2). This may result from competitive influences on the pheromone response. For example, the *daf* genes may affect response to both food and pheromone, and various levels of gene activity may affect each response to differing degrees. Also, we have observed that, in general, "sicker" animals form dauer larvae at a lower frequency than do animals that grow well. Strains carrying the *daf-4(m72)* nonsense allele grow more slowly and are less fertile than the reference mutant *daf-4(m63)*, and these secondary properties may influence the strain's quantitative response to the pheromone in our tests.

Both *daf-4* and *daf-7* animals express non-*ts* phenotypes that are suppressed in mutants with suppressible alleles. The simplest explanation for this is that a pleiotropic non-*ts* mutation produces multiple phenotypes, including the naturally *ts* dauer larva formation. However, a variety of other explanations are possible. For example, differential amounts of gene product or different domains of the product may be needed for the different processes.

The TSP is usually interpreted to represent the developmental time at which the mutant gene product is synthesized, assembled, or exerts its action (1). Such an interpretation is not valid for *ts* dauer-constitutive mutants, nor may it be valid in some other cases. For example, three *emb* genes produce *ts* mutants at high frequency, and all alleles of two of these genes are *ts* (27). This is unusual because *ts* missense alleles represent only about 1/25 of all mutations (25). Similarly, all alleles of *unc-83* are *ts* and may reflect a *ts* process revealed or induced by elimination of gene activity (28).

Six out of the seven dauer-constitutive mutants in this study are deficient in egg-laying, and at least two *egl* mutants, *egl-4* and *egl-40*, overrespond to the dauer-inducing pheromone. The egg-laying rate appears to be modulated by environmental factors such as the food supply, and some *egl* mutants may be defective in processing these environmental signals. The *egl* mutants have been classified into categories by their response to drugs that affect egg-laying (22). One category includes seven *egl* genes and four dauer-constitutive genes. All alleles of the seven *egl* genes in this category are *ts*.

One dauer-constitutive gene, *daf-2* is clearly different from the others. The *daf-2* gene holds a unique position in the genetic pathway for dauer larva formation, defining a "branch" that separates it from the other six dauer-constitutive genes (15). The *daf-2* mutants are not affected in egg-laying. At the permissive temperature, the *ts* reference strain CB1370 [*daf-2(e1370)*] did not overrespond to the pheromone as did other dauer-constitutive mutants; indeed, its response was less than that of wild type. Non-*ts* genetically lethal alleles of *daf-2* form dauer larvae regardless of temperature. These dauer larvae do not recover, so they do not reproduce. Thus, *daf-2* gene activity may be essential for bypassing the dauer-stage, and *ts* alleles of *daf-2* may simply be "leaky" alleles with partial activity (not necessarily thermolabile). The non-*ts* genetically lethal alleles may be null alleles.

The analysis of dauer-defective mutants indicates that the dauer-inducing pheromone is necessary for the formation of dauer larvae. All tested dauer-defective mutants failed to respond to the pheromone, suggesting that nonresponse to pheromone is essential for their phenotype. If pheromone were not necessary for dauer larva formation (i.e., if another cue, such as starvation, were sufficient in itself for the induc-

tion of dauer larvae), then some dauer-defective mutants might be expected to respond to pheromone if their defect only blocked the response to another cue. Also, a mutant deficient in pheromone production has been isolated recently, and the absence of pheromone production is sufficient to produce a dauer-defective phenotype. Addition of exogenous pheromone completely restores the mutant's ability to form dauer larvae (unpublished data).

The above observations lead to the following model for the sensory control of dauer larva formation in wild type and the *daf* mutants. In the wild type, the dauer-inducing pheromone and elevated temperature may act independently to enhance dauer larva formation, whereas food signal inhibits dauer larva formation. Normally, all three cues are simultaneously influencing the decision to form a dauer larva. The pheromone is necessary for dauer larva formation, and temperature and food signal have modulating effects. Dauer-defective mutants may have defects at any point in the signaling pathway, but they always underrespond to the pheromone. Some leaky mutants may exhibit temperature-dependence. Mutants that have lost all chemosensory function will be dauer-defective because responsiveness to pheromone is necessary for dauer larva formation in a wild-type genetic background. Dauer-constitutive mutants, on the other hand, produce a false, internal signal favoring dauer larva formation (15). This may result from a specific defect in their response to the food signal. In such a case, their overresponse to pheromone and temperature would be an indirect result of their genetic predisposition toward dauer larva formation. Temperature alone (25°C) becomes sufficient to induce constitutive dauer larvae, even in the complete absence of pheromone. At lower temperatures, their hyperresponse to pheromone becomes evident.

Further work will be required to understand the precise physiological effects of individual genes on the response of the animal to environmental factors. The set of *C. elegans* mutants affected in dauer larva formation may provide a unique opportunity for a detailed genetic analysis of pheromone production and response in a metazoan.

We thank Carol Trent and Louise Liao for providing their strains in advance of publication. Other strains were provided by the *Caenorhabditis* Genetics Center, which is supported by Contract NO1-AG-9-2113 between the National Institutes of Health and the Curators of the University of Missouri. This work was supported by Department of Health and Human Services Grant HD11239 and by Research Career Development Award HD00367 to D.L.R.; J.W.G. is a predoctoral trainee supported by Department of Health and Human Services Grant GM07494.

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