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Phenotypic and Genetic Analysis of a Mutant of *Heterorhabditis bacteriophora* Strain HP88[1]

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Abstract: Induction and characterization of a morphological mutant are described for *Heterorhabditis bacteriophora* strain HP88. A homozygous inbred line was used as the base population for mutagenesis and genetic analysis of mutations. Mutagenesis was induced by exposing young hermaphrodites to 0.05 M ethyl methanesulfonate. A dumpy mutant (designated *Hdpy-1*) was isolated from the F_2 generation of the mutagenized population. Morphological studies with light and scanning electron microscopy revealed that the head region of the adult stage was compressed. The head region of the infective juvenile was distorted and the mouth open. Backcross with the original population was successful only between mutant hermaphrodites and wild type males; 50–100 percent of the progeny of this cross maintained the dumpy phenotype, indicating that the ratio between self- and external fertilization of the eggs is >1 and that the dumpy mutation is recessive.

Key words: dumpy mutant, genetics, *Heterorhabditis bacteriophora*, mutagenesis, nematode.

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae have become in recent years important agents for biological control of insects (7,8). The infective stage of these nematodes seeks and invades larvae of a variety of soil-dwelling insect species, including some economically important pests (13).

Upon invasion, the nematodes release the symbiotic bacterium *Xenorhabdus* spp. into the hemolymph of the host, and the latter dies within 24–48 hours as a result of propagation of the bacteria and of the nematodes that feed on them. The virulence of these nematodes and associated bacteria, their lack of mammalian pathogenicity, and the availability of relatively simple and inexpensive methods for culturing them for commercial use have made them attractive candidates for biological pest control (8).

However, environmental extremes such as high temperatures, desiccation, and solar radiation reduce nematode efficacy considerably (6,12). One approach for overcoming these shortcomings is through genetic improvement (5), either by (i) selection of an existing variant displaying the desired trait(s) from a genetically heterogeneous wild-type population or (ii) induction in individuals from a wild-type population of mutations that confer the desired trait(s).

Heterorhabditis bacteriophora strain HP88 is very useful for genetic studies because it has a short generation time and is a self-reproducing hermaphrodite (4,10,16,17, 21). Recently, we have shown that this nematode can be propagated every generation from a single female by self-fertilization (10,21). Consequently, the inbreeding homozygous development of a wild-type population, or of a recessive mutation, from a single individual is automatic in the F_2 generation, and pure lines may be obtained and maintained easily (21). The availability of an appreciable proportion of males in the population (21) provides for the exchange of genetic markers, which is crucial for genetic analysis.

The objective was to screen for easily recognizable morphological or behavioral

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mutants of *H. bacteriophora* and to generate marker mutations useful for future genetic analysis and for the chromosomal assignment of desired mutations that confer beneficial traits. The present study describes the first successful induction of a morphological mutant in *H. bacteriophora* strain HP88 and its characterization.

Materials and Methods

Nematode culture and storage: The HP88 strain of *H. bacteriophora* was isolated in 1982 from infected June beetle larvae (*Phyllophaga* sp.; Scarabaeidae: Coleoptera) in Logan, Utah (17). Our original wild-type population was obtained from Biosys (Palo Alto, CA).

In vivo culture of the nematodes was done on the last instar of the greater wax moth *Galleria mellonella* (3). The nematodes were cultured in vitro in 5-cm-d petri plates on Dog Food Agar (DFA) (10) or NGM agar (NGM) (2). The DFA or NGM plates were preinoculated with the bacterium *Xenorhabdus luminescens,* associated with the HP88 nematode strain, which had been isolated and propagated according to Poinar and Thomas (18). The infective juveniles (IJ) were suspended in sterile saline solution, rinsed twice, and stored at 10 C in 0.1% formalin suspension in plastic culture flasks (15).

Generation of the 6Dy homogeneous wild-type line: A homozygous inbred line, coded "6Dy," was derived from the wild-type population with a procedure (10) in which each of 15 successive generations was initiated from a single virgin hermaphrodite, thus achieving a homozygotization value of >95%. However, because the 6Dy line could not be proven to be completely homozygous, we will refer to it as a wild-type homogeneous strain. The 6Dy line was used as the base population for mutagenesis and genetic analysis of mutations.

Mutagenesis: The following procedure was modified from protocols developed for the free-living nematode *Caenorhabditis elegans* (2): Fourth-stage juveniles and young hermaphrodites were collected from a parental generation, which was initiated from IJ 40–48 hours after IJ inoculation, at which time eggs are not yet present in their ovaries. This ensured virginity as well as the absence of males, which never develop from IJ (17,21). The nematodes were washed off the DFA plates in autoclaved M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$ in 1 liter distilled water) into a 15-ml conical tube. To 3 ml of this nematode suspension was added 1 ml of freshly prepared 0.2 M ethyl methanesulfonate (EMS, Sigma Chemical Co., St. Louis, MO) in M9 buffer (final concentration 0.05 M). The nematodes were kept in this mutagen solution for 2 hours at room temperature and allowed to settle by gravity. After concentrating the nematode suspension, 0.2–0.5 ml of it was transferred in droplets onto the surface of an NGM plate with a sterile pipette. The nematodes were maintained for 30 minutes on the agar to allow it to absorb an excess of mutagen solution. Mutagenized nematodes were washed off the NGM plate with M9 buffer, seeded on 5-cm-d DFA plates (100–200 nematodes per plate), and then incubated at 25–28 C until the young F_1 hermaphrodites appeared 4 days later. The F_1 virgin hermaphrodites were collected and seeded individually on 3-cm-d NGM plates, which were incubated for 4 days under the same conditions until young F_2-generation hermaphrodites had developed.

Screening and phenotypic analysis: Virgin F_2 hermaphrodites were washed off the NGM plates in M9 buffer into empty 5-cm-d plates and examined thoroughly with a stereomicroscope for morphological and behavioral abnormalities.

Putative mutant individuals identified in this way were each transferred to a new NGM plate to initiate a homozygous clone and to verify transmission of the mutant phenotype in subsequent generations. Confirmed mutants were further mass cultured on DFA plates. The IJ obtained from the DFA plates were surface sterilized with 0.85% (w/w) methylbenzethonium chloride (Sigma) solution for 10 min-

utes and then stored at 10 C in distilled water.

The sensitivity of mutant IJ to surface sterilization was examined by exposing them to 0.85% methylbenzethonium chloride for 15 minutes in 5-cm-d petri dishes and transferring them to distilled water in new petri dishes. The viability of IJ was recorded 1 hour later by observing their motility in the water suspension and their response to probing with a fine needle under a dissection microscope. A similar treatment was performed on IJ of the wild type (6Dy strain) as a control. Each treatment was replicated in eight dishes. The experiment was repeated twice.

Observations of head morphology and cuticular surface were made with a light microscope and with a scanning electron microscope (JFM 35-C SEM at ×1,000–6,000) following standard procedures (19).

Genetic crosses: Separate cultures of mutant and wild-type (6Dy) strains were initiated from IJ on DFA plates. Virgin hermaphrodites were collected as described above, and males were obtained from the F_1 generation by washing the DFA plates with M9 buffer. Some 15–20 males from one strain and a single virgin hermaphrodite from the other strain were placed together on a 3-cm-d NGM plate. Five days later, the adult progeny were available for phenotypic screening segregation analysis. This was conducted under a stereomicroscope. Each of the reciprocal crosses was replicated 15 times. In general, the yield of progeny per mating plate varied and was rather low (an average of 56 ± 37 progeny/plate).

Results and Discussion

Phenotypic characterization of the Hdpy-1 mutant: The mutagenesis yielded a "dumpy" mutant. In accordance with terminology used for *C. elegans* (19), we named this mutant *Hdpy-1*, where "H" stands for *Heterorhabditis*, "dpy" for the characteristic "dumpy" phenotype, and "1" for the first mutation in this locus.

The mutant nematodes had a dumpy shape rather than the more slender form of wild-type nematodes. The average length of the mutant hermaphrodites which developed from IJ on the DFA plates was 0.83 ± 0.15 mm (n = 73, range 0.57–1.14 mm). They were shorter ($P < 0.05$) than the wild-type nematodes (1.28 ± 0.32 mm, n = 79, range 1.20–1.73 mm) but maintained a similar width (0.05 ± 0.01 mm, n = 73, range 3.6–7.4 μm). The dumpy phenotype was apparent in the entire population, and the expressivity was 100%. It was detected at early stages of development (hatched first- and second-type juveniles), but its full manifestation was observed in the adults, both hermaphrodites and males.

The reduction in length of hermaphrodites of the F_1 generation as compared to hermaphrodites that develop from IJ, a feature typical of wild-type nematodes (16,21), was observed also in the *Hdpy-1* strain.

Examination with a scanning electron microscope revealed compression and foldings in the head region of the mutant adult hermaphrodites (Fig. 1B), whereas in the wild type this region was smooth (Fig. 1A).

It remains to be determined if *H. bacteriophora*-mated hermaphrodites produce more male progeny than self-fertilizing hermaphrodites, as is the case in *C. elegans* (20). If this were the case, then one explanation for the paucity of males in the mutant strain (6.2 ± 1.8% males in the mutant population compared to 12.7 ± 2.6% in the wild-type population) could be a reduced rate of cross-fertilization due to problems in mating as a result of the morphological abnormalities.

Mutant IJ morphology and cuticle formation: Scanning electron microscopy showed several morphological differences between the mutant IJ and their wild-type counterparts. Wild-type IJ had a closed mouth (Fig. 1C), whereas the mouth of the mutant IJ was partly open (Fig. 1D). In addition, the cuticle surrounding the mouth of mutant IJ was rough and had concentric wrinkles, which may represent initial signs

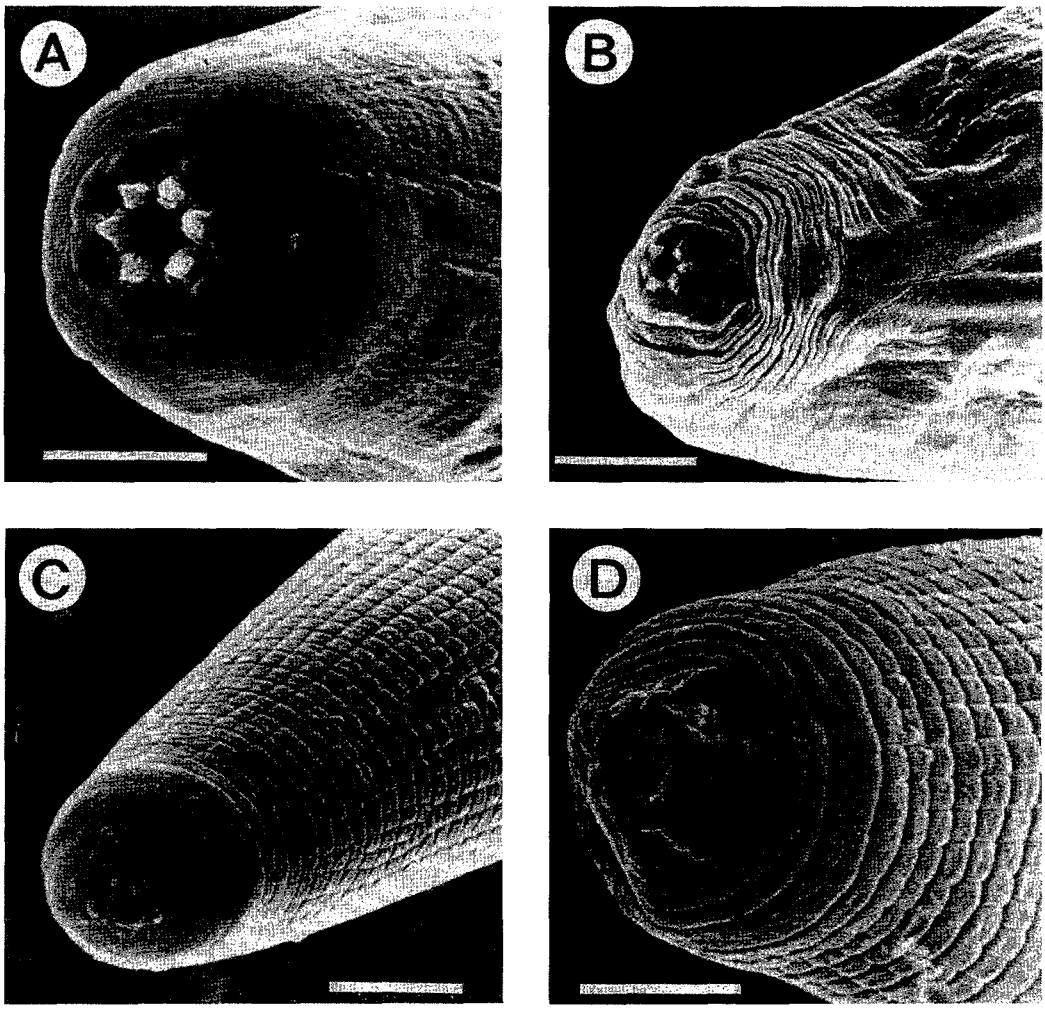

FIG. 1. Scanning electron photomicrographs of the anterior end of *Heterorhabditis bacteriophora* strain HP88. A) Normal hermaphrodite. B) Dumpy mutant of hermaphrodite. C) Normal infective juvenile. D) Dumpy mutant infective juvenile. Scale bar = 10 μm.

of the aberrations typical of the mutant adult head (compare Figs. 1A and 1B).

Infective wild-type juveniles (obtained from storage) were usually covered with two layers of cuticle (17). An outer one, that of the second juvenile stage, had numerous characteristic longitudinal striations (Fig. 2), under which was a layer of the third juvenile stage, with double paired lateral ridges. Similar lateral ridges were described for a number of steinernematid species (14).

In the mutant strain, approximately 50% of the IJ stored under the same conditions had only one cuticular layer, namely, that of the third juvenile stage, which has typical paired longitudinal ridges (Fig. 2).

Sensitivity to methylbenzethonium chloride: During preparation for storage, the IJ of the *Hdpy-1* mutant were found to be more sensitive to the detergent methylbenzethonium chloride than their wild-type counterparts. Exposure of the mutant IJ for 15 minutes resulted in 42.7 ± 12.3% reduction in IJ viability, whereas no reduction was recorded among the wild-type IJ. This detergent sensitivity may well result from

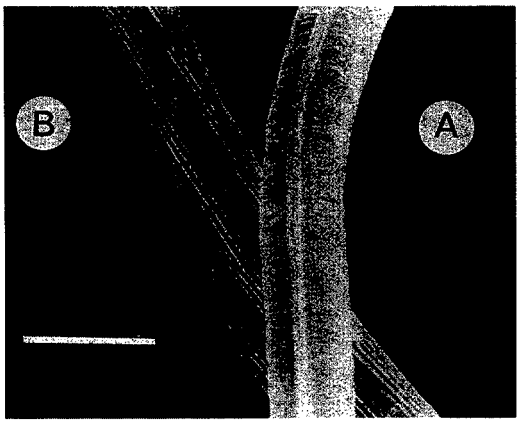

FIG. 2. Scanning electron photomicrographs of infective juveniles of *Heterorhabditis bacteriophora* strain HP88. A) Dumpy mutant treated with 0.85% Hymine for 15 minutes. B) Wild type, covered by the cuticle of the second-stage juvenile. Scale bar = 35 μm.

the above-mentioned cuticular abnormalities of the mutant and from the fact that its mouth is open.

Genetic characterization of the Hdpy-1 mutation: Fifteen reciprocal crosses were conducted between virgin individuals of the wild-type strain (6Dy) and the *Hdpy-1* mutant strain in an attempt to determine if the mutation is dominant or recessive, autosomal or X-linked.

Progeny were obtained from only eight crosses between wild-type males and mutant hermaphrodites, whereas the reciprocal crosses yielded none. Therefore, the results are only partially conclusive. It should be kept in mind that these crosses were conducted with relatively small numbers of individuals, and it is possible that matings involving a surplus of mutant males may yield progeny. The paucity of progeny in our crosses and the absence of males among them preclude any conclusion as to whether the *Hdpy-1* mutation is autosomal or X-linked.

Segregation of phenotypes was observed in the progeny of the cross of wild-type males to mutant females: dumpy nematodes always constituted 50–100% of the progeny (Table 1). Had the *Hdpy-1* mutation been dominant, we would have expected all the progeny of this cross to be

TABLE 1. Phenotypic diversity of generation progeny of *Heterorhabditis bacteriophora* (strain HP88) following crossing of males from a wild-type strain (6Dy) with dumpy mutant (Hdpy-1) hermaphrodites.

Plate	Normal	Dumpy	% Dumpy
1	3	3	50
2	12	15	55
3	1	7	87
4	43	172	80
5	0	6	100
6	18	32	64
7	0	28	100
8	35	76	68

Figures indicate number of progeny. Males (15–20) were placed with a single hermaphrodite on each DFA plate.

dumpy. Thus, the ratio obtained suggests that the *Hdpy-1* mutation is recessive. Moreover, these results also provide an estimate for the relative proportions of self- versus cross-fertilization. Exclusive self-fertilization in this cross would yield only mutant progeny, whereas exclusive cross-fertilization would give rise to normal progeny only. The preponderance of the mutant individuals observed among the progeny of this cross is thus compatible with <50% cross-fertilization.

It has been argued by Poinar and Georgis (17) that although the first-generation nematodes of *H. bacteriophora* 'HP88,' resulting from IJ, are hermaphrodites, reproduction in subsequent generations is amphimictic, requiring cross fertilization by males. In such a case, the value of this nematode species for genetic research would be reduced because no automatic homozygotization would be possible as in *C. elegans*. However, the fact that we were able to generate homogeneous lines in a previous study (10) and the 6Dy line in the present report by serial subculturing of single hermaphrodites for 15 generations in the absence of males suggests that *H. bacteriophora* can be propagated exclusively through self-fertilization of hermaphrodites. Furthermore, the availability of a genetic marker, the *Hdpy-1* mutation, provided a means for demonstrating that cross-fertilization is possible in every generation if males are available. This is evi-

dent from the observation that when mutant hermaphrodites, developed from IJ, were crossed with wild-type males, approximately half the progeny were of wild-type phenotype and approximately half were dumpy, indicating that self- as well as cross-fertilization occurred under our experimental conditions.

Reproductive patterns may be affected by rearing conditions. In the present research, the nematodes were cultured in vitro on a rich artificial medium (DFA) and were capable of self-fertilization. In a previous study (17), however, nematodes that were reared in vivo on larvae of the greater wax moth *G. mellonella* showed enhanced development of males (cf. our study) and amphimictic reproduction.

Our success at obtaining a mutant indicates that in principle, the methodology developed for EMS mutagenesis in the free-living nematode *C. elegans* is applicable to other nematodes, particularly for steinernematids and heterorhabditids. This should allow us to improve beneficial traits such as tolerance to desiccation and solar radiation, as well as host-finding ability, which could be better improved by generating mutations that will enhance them, rather than by selection (10).

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