Species Differentiation in Caenorhabditis briggsae and Caenorhabditis elegans

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Abstract: Identification of five laboratory strains (1-5) of putative *Caenorhabditis briggsae* was undertaken. Examination of the male bursal ray arrangement, mating tests with males of *Caenorhabditis elegans*, malate dehydrogenase zymograms, and SDS polyacrylamide electrophoresis demonstrated that strain 4 was *C. briggsae* and the others were *C. elegans. Key Words:* bursal ray arrangement, mating test, malate dehydrogenase, SDS polyacryamide electrophoresis, morphology, taxonomy.

Two free-living nematodes, *Caenorhabditis briggsae* (Dougherty and Nigon, 1949) Dougherty, 1953 and *C. elegans* (Maupas, 1900) Dougherty, 1953 have become important model systems for biological studies and have been used extensively for biochemical (14), nutritional (13), genetic (2, 6), neurobiological (17), and aging (14, 18) research. The basic biology of both nematodes was described by Nigon and Dougherty (12). These species provide a unique opportunity to study the biology of closely related nematode species in culture.

We have been investigating drug action and nutritional physiology in two strains of *Caenorhabditis* that supposedly were representative of *C. briggsae* and *C. elegans.* However, the nutritional requirements of the *C. briggsae* strain did not correspond to those reported by Hansen and Buecher (7). To resolve this discrepancy, other laboratory strains of *C. briggsae* were obtained for the comparative studies presented herein.

The specific morphological separation

of *C. briggsae* and *C. elegans* is based mainly on the arrangement of the bursal rays in the male. However, species identification is impeded by the rare occurrence of males in cultures of these hermaphroditic nematodes (12). To overcome these difficulties, electrophoretic analysis of malate dehyrogenase and proteins was undertaken in combination with morphological, genetic, and nutritional studies to differentiate *C. briggsae* and *C. elegans.*

MATERIALS AND METHODS

Nematode strains: *Caenorhabditis elegans* variety Bristol was obtained from two sources: *C. elegans,* code name N2, in monoxenic culture with *Escherichia coli* from Dr. R. L. Russell at the California Institute of Technology; *C. elegans* in axenic culture from Dr. E. L. Hansen at the Clinical Pharmacology Research Institute, Berkeley. The five laboratory cultures of *C. briggsae* in axenic cultures were obtained from five laboratories located in the United States and Australia and designated strains 1-5.

Maintenance media: *C. elegans* N2 had abundant males and was maintained on NG agar (2) with wild type *E. coli.* The axenic nematodes were maintained in *Caenorhabditis briggsae*-maintenance medium (4), supplemented with 200 μg cytochrome *c*/ml, and 50 μg β-sitosterol/ml (=ACM) (14) or ACM supplemented with 25 mg lactalbumin hydrolysate/ml (=LCM). Five ml of media were placed in 50-ml Erlenmeyer flasks and inoculated with 50-500 nematodes/ml. The nematodes were usually harvested after 10 days of growth at 20 ± 0.5 C.

Mating test and morphology: Crosses of males of *C. elegans,* N2, were attempted with hermaphrodites of all strains of *C. briggsae* and *C. elegans* according to the methodology of Dusenbery et al. (6). Successful crosses were recognized by the postmating abundance of males. Males were rarely observed in cultures other than N2. Nematodes in axenic cultures were transferred to NG agar with lawns of *E. coli* and maintained in monoxenic culture for 1-2 months (weekly transfers) before the mating test.

The arrangement of the bursal rays in the male nematodes was observed on males anesthetized with propylene phenoxetol (8) in 0.1 M NaCl. Photographs were taken with Nomarski interference optics on a Zeiss Photomicroscope III.

Nematode homogenates: Nematodes from axenic cultures were harvested by a modification of the method of Sulston and Brenner (15). After the final centrifugation at 4 C for 5 min at 500 g, the nematode pellet was resuspended in 0.01 M potassium phosphate (pH 7.0) to yield a 20% suspension (v/v) of nematodes. The nematodes were homogenized in an Aminco French pressure cell at 705 kg/cm². The homogenate was collected and centrifuged at 35,000 g for 30 min at 4 C.

Supernatants for isozyme analysis were stored in 200-μ liter samples at −20 C. Prior to electrophoresis, the samples were thawed, centrifuged at 31,000 g for 1 min in a Beckman Microfuge B, and the amount of protein in the preparation was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard.

Supernatants for SDS (sodium dodecyl sulfate) protein electrophoresis were assayed for protein concentration and subsequently boiled for 4 min in 77 mM Tris-HCl (pH 8.0), 1.77% SDS, 4.41% MCE (mercaptoethanol), and 3.0% glycerol. The protein preparations were divided into 200-μliter samples and stored at −20 C (5).

Electrophoresis: Isozymes of malate dehydrogenase (MDH; EC 1.1.1. 37) were separated by vertical starch gel electrophoresis (3). The gel buffer system was 3.5 mM citric acid and 9.7 mM sodium phosphate (dibasic). The electrode buffer was 5.25 mM citric acid and 14.5 mM sodium phosphate (dibasic). The buffer solutions were adjusted to pH 7.0. Samples (50 μl) containing 190 μg of protein were placed in each well. Electrophoresis was conducted at 150 volts for 19 h at 4 C. The gel was sliced, and half was incubated in the MDH specific stain (3) for 2 h at room temperature in the dark. After being stained, the gel was rinsed in distilled water and preserved in 50% ethanol. The remaining half of the gel was incubated in staining solution prepared without malic acid.

SDS-soluble proteins were separated in a discontinuous system by vertical slab electrophoresis in polyacrylamide SDS gels by the method of Conejero and Semancik (5). Samples were applied at a concentration of 20 μg/sample well. Electrophoresis was conducted in a Hoefer SF 500 Vertical Slab Electrophoresis assembly at 20 ma/slab in an anionic system at 4 C for 2-2.5 h. After electrophoresis, the gels were fixed overnight in 12.5% TCA (W/V), stained for 3 h in 0.8% Coomassie brilliant blue G, and destained with water. The following proteins were electrophoresed along with the sample homogenates for molecular weight determinations: bovine serum albumin (69,000), ovalbumin (43,000), trypsin (23,000), and cytochrome *c* (11,700).

Enzyme assay: MDH activity was determined by measuring the absorbance decrease at 340 nm in a Gilford spectrophotometer at 25 C. The standard assay contained 50 mM potassium phosphate (pH 7.0), 0.1 mM NADH, and 0.25 mM oxaloacetate in a final volume of 1.0 ml. The reaction was initiated by the addition of enzyme, and absorbancy changes were recorded for 2 min. The molar extinction coefficient of 6.2×10^{-3} (1) was used to calculate the specific activity of the enzyme.

RESULTS

Glucose requirement: The growth of three strains of *C. briggsae* was studied in ACM with and without glucose (Table 1).

TABLE 1. Comparative growth of three putative strains of *Caenorhabditis briggsae* in CbMM[a] without glucose.

Strain	Growth as % of control at sampling interval[b]		
	1	2	3
1	93	73.4	88.2
2	119	82.7	76.9
4	110	113	99.4

[a]*Caenorhabditis briggsae* maintenance medium supplemented with 50 μg β-sitosterol/ml and 200 μg cytochrome *c*/ml.
[b]The sampling times for strains 1 and 2 were 6, 13, and 20 days, whereas those for strain 4 were 7, 14, and 21 days. Each strain was tested in separate experiments and each experiment consisted of six control and six minus glucose flasks.

Growth in the absence of glucose was similar to growth of the control during the first week in strains 1 and 2. Thereafter, the growth of both strains was lower than that of the controls during the remainder of each experiment. Strain 4 grew better than the control during the first 2 weeks and then at the same rate as the control during the third week. The other nematode strains were obtained later in this study, and growth in glucose deficient ACM was not studied.

Morphology and mating test: Examination of the male tail of *C. elegans,* N2, showed the bursal ray arrangement (Fig. 1-A) (16) described by Nigon and Dougherty (12) for *C. elegans.* This arrangement is characterized by the large separation between the first pair of postanal rays and the grouped, second to fourth pairs of postanal rays. Spontaneous males of *C. briggsae* strains 1-3, and 5 and *C. elegans* from Dr. Hansen occurred in monoxenic cultures and the arrangement of the bursal rays was the same as that shown in Fig. 1-A. Spontaneous males of *C. briggsae* strain 4 occurred once in axenic culture in LCM and seven were obtained. The arrangement of the bursal rays was studied in two males and two patterns were observed (Fig. 1-B, C). Although the nematode in Fig. 1-B had the same total number of pairs of bursal rays as *C. elegans,* the first postanal pair was not separated from the second to fourth postanal pairs. The nematode in Fig. 1-C showed an alternate arrangement: apparent fusion of the first and second postanal pairs occurred on one side. Both patterns seen in this work were described by Nigon and Dougherty (12) for *C. briggsae.* The remaining five males were placed in monoxenic culture with five hermaphrodites of strain 4 in an attempt to develop a male-producing line of *C. briggsae.* The nematodes did not mate and the males were lost.

Hermaphrodites differed only in the number and arrangement of the eggs in the uterus. In *C. elegans,* numerous eggs (6 to 20) were present, and the long axis of the eggs was transverse to the long axis of the nematode; in *C. briggsae,* small numbers of eggs (1-6) were present, and the long axis of the egg was parallel to the long axis of the nematode.

FIG. 1-(A-C). Arrangement of bursal rays [after Cobb's formula (16, p. 449)]. *A) Caenorhabditis elegans,* N2, bursal ray formula (BRF) = 2 () 1-3-3. *B) C. briggsae,* strain 4, BFR=2 () 4-3. *C) C. briggsae,* strain 4, BRF=2 () 3, 4-3.

Mating tests between the males of *C. elegans,* N2, and hermaphrodites of *C. briggsae* strains 1-3, 5, and *C. elegans* were successful (Table 2). The arrangement of the bursal rays was examined in the male progeny and it is illustrated in Fig. 1-A. Strain 4 hermaphrodites did not mate with *C. elegans* males (Table 2).

Electrophoresis. Two major isozymes of MDH, one cathodic and one anodic, were separated by starch gel electrophoresis (Fig. 2). This pattern was identical for *C. briggsae* strains 1-3, and 5 and *C. elegans.* The cathodic and anodic isozymes of *C. briggsae* strain 4 (a-d) differed in mobility

from those of *C. briggsae* strains 1-3, and 5 and *C. elegans.* The 4a and 4b samples were prepared from the same actively growing culture in which half of the tissue volume consisted of eggs. The eggs and nematodes
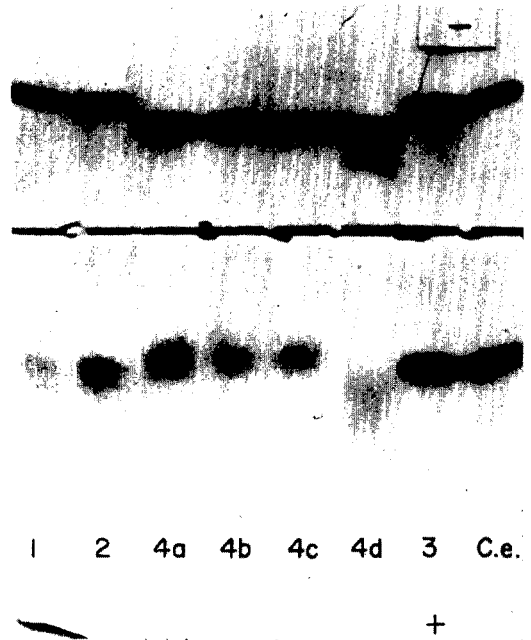
TABLE 2. Bursal ray arrangement and mating in strains of *Caenorhabditis elegans* and *C. briggsae* in monoxenic culture.

Strain	Male tail pattern	Mating with N2 male
Caenorhabditis elegans		
N2	ce[a]	+
Hansen	ce	+
Caenorhabditis briggsae		
1	ce	+
2	ce	+
3	ce	+
4	cb[b]	—
5	ce	+

[a] *Caenorhabditis elegans* pattern.
[b] *Caenorhabditis briggsae* pattern.

FIG. 2. MDH isozymes of *Caenorhabditis briggsae* strains 1-4 and *C. elegans* (C.e.) separated by starch gel electrophoresis: 4a — nematode homogenate with low number of eggs; 4b — homogenate with high number of eggs; 4c — homogenate with high number of eggs; 4d — homogenate of "old" nematodes.

were separated by placing the culture on wet-strength tissue supported by wire gauze in a glass petri dish. After 2 h, the nematodes which had migrated through the paper were collected (sample 4a). Although a number of eggs passed through the tissue, the resulting egg sample (4b) collected from the upper part of the Baermann apparatus contained 5 times the number of eggs as sample 4a. Sample 4c was from another actively growing culture with high numbers of free eggs (half of the total tissue volume used in homogenization). Such high concentrations of eggs were never seen in the other nematodes cultured under the same conditions. Sample 4d was prepared from a culture in stationary growth and a supplemental MDH cathodic isozyme was found. The anodic isozyme migrated in a diffuse manner (Fig. 2). Equal amounts of samples 4b and 1 were mixed and run on starch gel in two experiments. Both anodic and cathodic isozymes of both nematode strains separated under these conditions, a demonstration that these proteins had unique electrophoretic mobilities.

These findings were substantiated by vertical slab polyacrylamide electrophoresis. Four-six MDH isozymes were found in all strains, with *C. elegans* and *C. briggsae* strains 1-3, and 5 isozymes exhibiting identical electrophoretic mobilities (E_f). All the cultures of *C. briggsae* strain 4 exhibited isozyme patterns with greater E_f. Differences were also found between the actively growing and stationary phase strain 4 samples.

The SDS soluble proteins of *C. elegans* and *C. briggsae* strains 1-5 were compared by polyacrylamide electrophoresis and 50-60 protein bands were observed (Fig. 3). The protein patterns of *C. elegans* and *C. briggsae* strains 1-3, and 5 were identical. The protein pattern of *C. briggsae* strain 4 differed from all other strains qualitatively and in respect to the relative concentrations of certain proteins. Differences in protein patterns were noticed at the following molecular weights: 15,000-20,000; 23,000; 50,000; and 60,000-70,000. Quantitative differences were also observed between the different homogenates of *C. briggsae* strain 4 in the 15,000-30,000 molecular weight range.

MDH assay. The specific activity of MDH in the homogenates used for starch gel electrophoresis was 4.2 ± 0.4 (SE) moles NADH oxidized per minute per mg protein for *C. elegans* and *C. briggsae* strains 1-5.

DISCUSSION

Our results have shown that the morphology of males of *C. briggsae* strain 4 (obtained from Dr. Bert Zuckerman, University of Massachusetts, Wareham) conformed to that described for the species by Nigon and Dougherty (12). In contrast, the other strains, 1-3 and 5, received as putative *C. briggsae* were actually *C. elegans.*

The hermaphrodite characters, i.e., number and positioning of eggs in the uterus, vary according to the nutritional
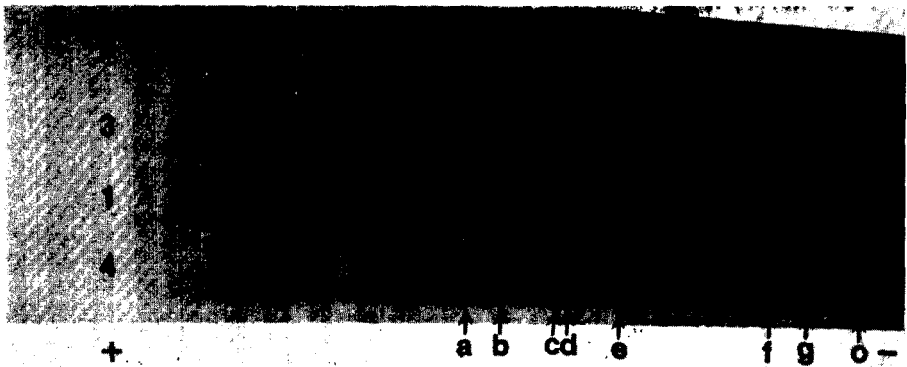

FIG. 3. Protein patterns of *Caenorhabditis briggsae* strains 1-4 separated by SDS polyacrylamide electrophoresis (vertical slab). The labeled regions indicate major differences between strains 4 and 1-3: (a-d) 15,000-20,000 mol. wt.; (e) 23,000 mol. wt.; (f) 50,000 mol. wt.; (g) 60,000-70,000 mol. wt.; (o) origin.

status of the nematodes (12). Low egg production in *C. elegans* may result in low numbers of eggs in the uterus, and the uncrowded condition allows the egg orientation to revert to the pattern typical of *C. briggsae*. Therefore, these characters are not reliable for species differentiation.

Satisfactory differentiation of these species was also achieved by the mating test (6, 12), but the usual absence of males precludes both morphological and mating tests under most laboratory conditions. Although the morphological and mating compatability evidence was definitive for the specific identification of these nematode strains, the need for alternative methods was apparent. We have demonstrated that electrophoresis can separate the strains of *C. briggsae* into the correct species. Electrophoresis of nematode proteins has shown that characteristic enzyme and protein profiles exist at the species level (9). The finding of markedly different MDH isozyme profiles and differences in proteins between *C. briggsae* strains 1-3, and 5 and *C. briggsae* strain 4 further supports our conclusions.

Our results have also shown that certain differences in isozyme and protein profiles existed between homogenates of *C. briggsae* strain 4. These differences were probably age related, although we have not proven this. The presence of "altered" (reduction in specific activity) enzymes has been demonstrated in "aged" *Turbatrix aceti* (14). However, changes in isozyme patterns of aged nematodes have not been observed in the enzymes studied to date.

The difference in glucose requirement of *C. briggsae* and *C. elegans* as described by Hansen and Buecher (7) was not confirmed. Although the experimental conditions were different, *C. briggsae* (strain 4) was independent of glucose in the medium, and the growth of *C. elegans* (strains 1 and 2, *C. briggsae*) was affected slightly. Currently, identification by nutritional tests requires further study.

Interspecies cell contamination has been discovered many times in cell cultures that were widely used, and it is not surprising to find that the identification of nematodes widely used for nutritional and biochemical studies has become confused. It is of importance to sort out these differences because biochemical discrepancies have appeared in studies on *C. briggsae* (11).

The taxonomic approaches, morphology and mating tests, have clarified the status of five strains of *C. briggsae*. However, these approaches are difficult and time consuming with hermaphroditic species. In this respect, new approaches should be examined and implemented as needed. Biochemical systematics may be the answer to these types of taxonomic problems. In the case of *C. briggsae* and *C. elegans*, nutritional physiology was not a satisfactory method for species identification. However, our findings should not preclude the use of nutritional physiology in the identification of other closely related nematodes. In contrast, electrophoretic analysis of proteins and isozymes provided conclusive evidence that closely related nematodes can be identified to the level of species on the basis of these biochemical properties. Similarly, polyacrylamide electrophoresis of proteins and isozymes has been used as an aid in the taxonomy of certain species complexes of plant-parasitic nematodes (9).

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