Characterization of dsRNA in *Chalara elegans* and Effects on Growth and Virulence

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ABSTRACT

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Forty-three isolates of *Chalara elegans* (synanamorph *Thielaviopsis basicola*) from 10 geographic locations within the United States, Canada, New Zealand, and the Netherlands were examined for the presence of double-stranded ribonucleic acid (dsRNA). Thirty-six of the isolates contained dsRNA elements and were assigned to nine groups based on the similarity of banding patterns after agarose gel electrophoresis. Hybridization experiments revealed some homology between one dsRNA segment (2.8 kb) in eight isolates. Isometric virus particles approximately 47 nm in diameter were observed in one isolate. Representative isolates from five main dsRNA groups were compared for differences in growth rate, sporulation, mycelial dry weight production, and virulence. A significant difference ($P < 0.05$) was observed in growth rate and virulence between isolates that contained dsRNA and those that did not. Within the dsRNA-containing isolates, only isolates in group C (with six dsRNA segments) were significantly different from the other isolates. During this study, a spontaneous change in phenotype was observed in three isolates; dsRNA analysis indicated that the banding patterns had been altered from multiple segments to either a single 2.8-kb segment (two isolates) or to no dsRNA present (one isolate). A corresponding increase in growth rate or virulence was associated with these dsRNA changes in the two partially cured isolates. However, isolates in which the 2.8-kb fragment was either eliminated (cured) or was initially absent were the least virulent. This putative association of a 2.8-kb dsRNA fragment with enhanced virulence requires further study. The results from this research show that the presence of dsRNA in *C. elegans* is ubiquitous and can alter fungal physiology and virulence. A reduction in growth rate and virulence due to the presence of multiple dsRNA in some isolates of *C. elegans* suggests that survival and pathogenicity can be affected in this soilborne facultative parasite.

Additional keywords: black root rot, fungal viruses, hypovirulence.

The soil-inhabiting dematiaceous hyphomycete *Chalara elegans* Nag Raj & Kendrick (synanamorph *Thielaviopsis basicola* Berk. & Broome) Ferraris (20) is associated with the roots of a wide range of plants in nature (37) and is recognized as a pathogen that causes black root rot on many important agricultural crops worldwide (10,17–19,26). The organism is a facultative parasite that generally infects seedlings or stressed plants and frequently colonizes roots as a secondary invader (17–19,26). Differences in pathogenicity among isolates of *C. elegans* have been reported previously (15,17,32); in addition, there can be marked morphological differences among isolates in culture (15). The basis for these differences is unknown, although the numerous morphological variants that arise in culture are thought to be the result of frequent mutation events (15).

In 1977, Bozarth and Goenaga (4) reported the occurrence of double-stranded ribonucleic acid (dsRNA) elements and virus-like particles (VLPs) in the cytoplasm of one strain of *C. elegans*. DsRNA elements have since proven to be widespread in fungi; frequently, however, a visible or quantitative effect on the fungal host cannot be demonstrated (5,22). Furthermore, it can be difficult to transfer the dsRNA between fungal strains to demonstrate an alteration of the phenotype or physiology of the fungus. The most well-described fungus is *Cryphonectria parasitica* and its hypovirulence-associated dsRNA elements (1,6,7,9,13,14,16,23, 29,30). Other economically important pathogens that contain dsRNA include *Sclerotinia sclerotiorum* (3), *Pythium irregulare* (11), *Phytophthora infestans* (21,33), and *Rhizoctonia solani* (2). In some of these fungi, the presence of dsRNA affects growth and virulence (3,33) or has no measurable and consistent effect (2).

Since the report by Bozarth and Goenaga (4), there have been no studies to elucidate the significance of dsRNAs in *C. elegans*.

The objectives of this study were to determine the prevalence of dsRNA within *C. elegans* and to study the correlations between the presence of dsRNA elements and the observed differences in morphology, growth, or virulence. A total of 43 isolates from 10 geographic regions were included in this study, of which four were reference cultures from the American Type Culture Collection (ATCC). Preliminary results from this study have been published (25).

MATERIALS AND METHODS

Fungal strains. The 43 isolates of *C. elegans* used in this study (Table 1) were obtained from different geographic areas and substrates. They were grown on V8 agar (V8 juice, 150 ml; Bacto agar, 15 g; distilled water, 850 ml; ampicillin, 100 mg) on the laboratory bench (23–27 C). Subcultures were made every 3–4 wk by transferring a 7-mm-diameter plug from the colony margin onto fresh V8-agar plates. All isolates were confirmed to be *C. elegans* by the presence of dark, thick-walled chlamydospores and hyaline phialospores (20).

Extraction and purification of dsRNA. Two mycelial plugs (7 mm in diameter) of each isolate were taken from the margin of 1- to 2-wk-old colonies and transferred to 20 ml of half-strength potato-dextrose broth (PDB, 12g/L, Difco Laboratories, Detroit, MI). Cultures were maintained at room temperature for 3–4 days, after which the plugs were removed and the fungal mat was collected by centrifugation at 20,000 *g* (9,000 rpm) for 10 min. The mats were washed with 0.1 M NaCl and homogenized in 5 ml of buffer (50 mM Tris HCl, pH 8; 20 mM EDTA; 2% SDS [sodium dodecyl sulfate]) with a hand-held homogenizer. Proteinase K (100 μg/ml; Sigma Chemical Co., St. Louis, MO) was added, and the solution was incubated at 55 C for 1–2 h with shaking. After chilling on ice, saturated NaCl (6 M) was added (0.35 ml/ml). The suspension was mixed by gently inverting and then centrifuged at 5,300 *g* (5,000 rpm) for 20 min. The resulting supernatant was carefully transferred to fresh tubes with wide-bore pipettes and was ethanol-precipitated overnight at −20 C. The precipitated material was collected by centrifugation for 10 min at 5,300 *g* (5,000 rpm), washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris; 1 mM EDTA). At this point, the preparation was treated with RNase (Boehringer Mannheim Canada Ltd., Quebec; ribonuclease A, DNase-free, 1 mg/ml of stock) at a final concentration of 25 g/ml in the sample with 0.3 M NaCl. The solution was incubated at 37 C for 10 min and precipitated by adding 0.8 volume of isopropanol and placing at −20 C from 2 h to overnight. The final pellet was washed with 70% ethanol, resuspended in TE buffer, and stored at 4 C.

Agarose gel electrophoresis of dsRNA. The dsRNA samples were separated in 0.7% agarose gel on a 6-cm-long horizontal minigel apparatus (Fisher Scientific, Nepean, ON, Canada) run for 2–3 h. The gel was prepared with 1× TBE buffer (89 mM Tris base; 89 mM boric acid; 2.5 mM Na_2EDTA) and contained 1 μg of ethidium bromide per milliliter. Electrophoresis was carried out at 3 V/cm. Nucleic acids were visualized on a UV transilluminator and photographed through an orange filter with Kodak Plus-X pan film type 4147. The sizes of the dsRNA were calculated by visual comparison with electrophoretic mobilities of a 1-kb dsDNA ladder (Gibco/BRL, Burlington, ON, Canada).

Confirmation of dsRNA. To confirm the double-stranded nature of the RNA, enzymatic digestions of nucleic acid samples were performed with DNase (7.5 units) at 37 C for 10 min and also with RNase (25 μg/ml) in both high-salt (0.3 M NaCl) and low-salt (0.03 M NaCl) buffers at 30 C for 1 h. Nucleic acids that were hydrolyzed by the RNase in low-salt but not in high-salt buffer and that were resistant to DNase were considered dsRNA (13). The presence of dsRNA also was shown by CF11 column chromatography (34), modified as follows. The nucleic acid sample was precipitated with 0.1 volume of 3 M NaOAc and 2 volumes of 95% ethanol, resuspended in 1× STE buffer (0.1 M NaCl; 0.05 M Tris; 1 mM EDTA, pH 6.8), and applied to a 6-cm column of CF11 cellulose (Whatman; Anachemia Science, Montreal) that had been prewetted with wash buffer (16.5% ethanol in 1× STE buffer). Approximately 3 ml of wash buffer was added immediately, and the resulting fraction of unbound material was collected. The dsRNA was eluted by adding 3 ml of 1× STE buffer, ethanol-precipitated at −20 C overnight, and pelleted by centrifugation for 15 min at 15,850 *g* at 4 C. The dsRNA was resuspended in TE buffer and stored at 4 C. Electrophoresis conditions were as described above.

TABLE 1. Isolates of *Chalara elegans* included in this study

Isolate[a]	Geographic origin	Substrate	Source
AK: 4N1, 89-1, 89-2, 89-3, 89-4	Clarkedale, AK	Cotton, 1989	C. Rothrock
AK: 89-6, 89-7, 208, 308	Hope, AK	Cotton, 1989	C. Rothrock
BCA, BC92, BC92CO	Cloverdale, BC	Carrot, 1990, 1992	Z. Punja
BK: 16-1, 16-2, 18, 25, 28, 34-2, 38-1	Kings County, CA	Cotton soil, 1991	B. Holtz
BK: 101	Monterey County, CA	Cyclamen, 1991	B. Holtz
BK: 102, 103	Kings County, CA	Cotton root, 1992	B. Holtz
CA1	Kern County, CA	Carrot, 1990	Z. Punja
F374	Kern County, CA	Cotton field, 1989	J. Marlow
FL-W	Dade County, Florida	Carrot, 1989	Z. Punja
HA	Amsterdam, Holland	Carrot, 1990	Z. Punja
NC1	Allegheny, NC	Tobacco roots, 1988	D. Shew
NC2	Watauga, NC	Tobacco roots, 1988	D. Shew
NC1526	Mitchell, NC	Tobacco roots, 1987	D. Shew
NC1527	Madison, NC	Tobacco roots, 1987	D. Shew
NC17	Haywood, NC	Tobacco soil, 1992	D. Shew
NC30	Madison, NC	Tobacco soil, 1992	D. Shew
NC49	Buncombe, NC	Tobacco soil, 1992	D. Shew
OR1	Texas	Vinca, 1988–1989	J. Marlow
OR2	Clackomas County, OR	Petunia, 1991	J. Marlow
TX	West Texas	Cotton, 1991	K. Arthur
WASH	Bellingham, WA	Carrot, 1989	Z. Punja
ATCC9109	North Carolina	Tobacco roots[b]	E. E. Clayton
ATCC9853	New Zealand		J. A. Stevenson
ATCC18722	North Carolina	Tobacco, 1969	D. F. Bateman
ATCC34114			G. C. Papavizas
CKP	Pullman, WA	Chickpea seed, 1993	W. Kaiser
HRB	Cloverdale, BC	Motherwort, 1993	P. Morris

[a] Original strain numbers as designated by collector.
[b] Year unknown.

Relationships between dsRNAs: Preparation of dsRNA probe. A probe was constructed from a 2.8-kb dsRNA segment from isolate CA1 (Table 1) that was easily purified by column chromatography as outlined above. A nonradioactive system incorporating digoxigenin-labeled dUTP (DIG-dUTP) was used. Approximately 50 ng of dsRNA was combined with random primers (100 ng, nine bases in length) in a 0.5-ml polypropylene tube and heated in boiling water for 5 min. After briefly chilling on ice, the following components were added: 1× reverse transcriptase reaction buffer (Gibco/BRL); 10 mM DTT; 200 M each of dATP, dGTP, and dCTP; 25 M dTTP; 12.5 M DIG-dUTP (Boehringer Mannheim), and sterile distilled water to give a final volume of 19 μl. The tube was gently mixed, centrifuged very briefly, and incubated at 37 C for 2 min to equilibrate. One microliter (=200 units) of M-MLV H^- reverse transcriptase (Superscript, Gibco/BRL) was added, and the mixture was returned to the 37 C water bath for 1 h. The reaction was terminated by adding 4 μl of 0.5 M EDTA. The resulting nucleic acid was precipitated overnight with LiCl following the protocol of the DIG-dUTP manufacturer (Boehringer Mannheim). The probe was stored at −20 C.

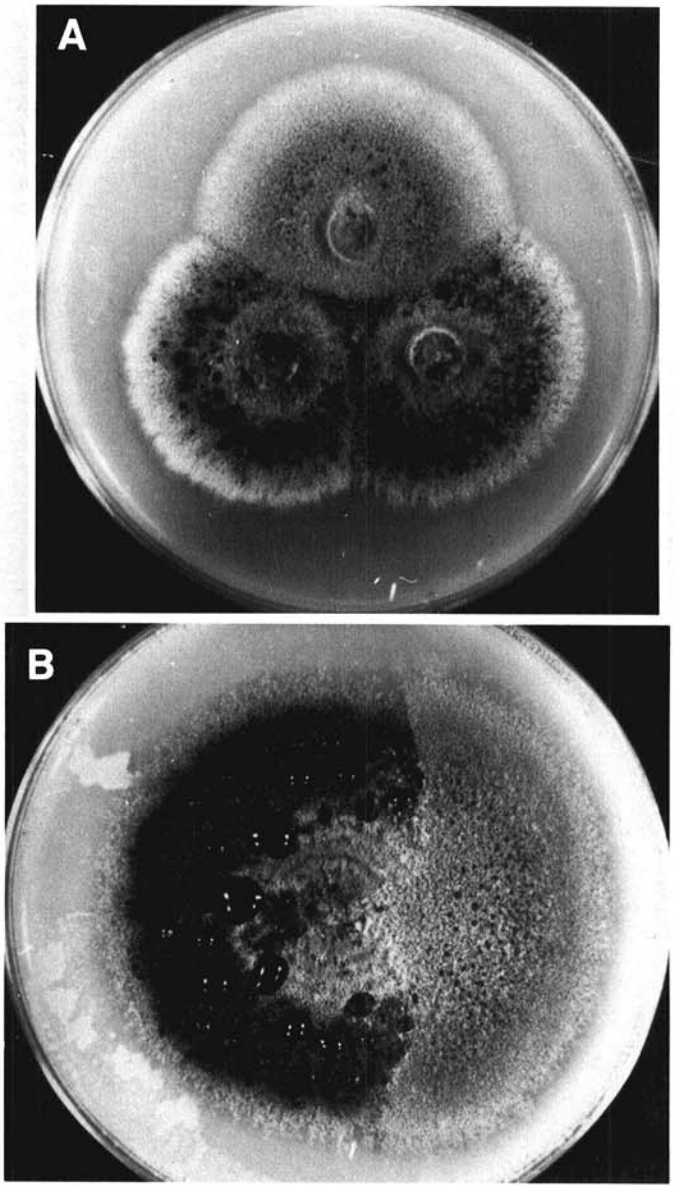

Fig. 1. Morphological variation in *Chalara elegans*. **A,** Isolate CA with group A morphology (top); isolate BK28 with group B morphology (lower right); isolate NC1 with group C morphology (lower left). Isolates were grown on V8 agar at 23 C for 7 days. **B,** Sectoring in isolate NC1 culture with altered morphology on the right and original culture morphology on the left.

DsRNA blotting and hybridization. After electrophoresis, the gel was incubated in depurination solution (0.5 M HCl) for 5 min, followed by denaturing solution (0.05 M NaOH, 0.01 M NaCl) for 30 min and neutralizing solution (0.1 M Tris, pH 7.5) for 30 min. After rinsing briefly in distilled water, the dsRNA/DNA was blotted by capillary action onto a Gene bind nylon membrane (Pharmacia P-L Biochemicals, Inc., Milwaukee, WI) in 10× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) for 3 h and cross-linked under ultraviolet light (Stratagene Crosslinker; Stratagene Cloning Systems, La Jolla, CA). The membrane was prehybridized with gentle shaking at 68 C for 12–24 h, hybridized with 20 ng of probe with gentle shaking at 68 C for 12–24 h, rinsed under high-stringency conditions (2× SSC + 0.1% SDS, 2 × 5 min, at room temperature; 0.1× SSC + 0.1% SDS, 2 × 15 min, at 68 C), and subjected to chemiluminescence detection as outlined by Boehringer Mannheim. The hybridization buffer was collected and stored at −20 C for re-use. The probe also was tested at low stringency (2× SSC + 0.1% SDS, 2 × 15 min, at room temperature; 2× SSC + 0.1% SDS, 2 × 15 min, at 42 C).

Mycelial growth and sporulation. A minimum of three to four isolates of *C. elegans* that contained dsRNA fragments (determined from the studies mentioned) representing a unique dsRNA

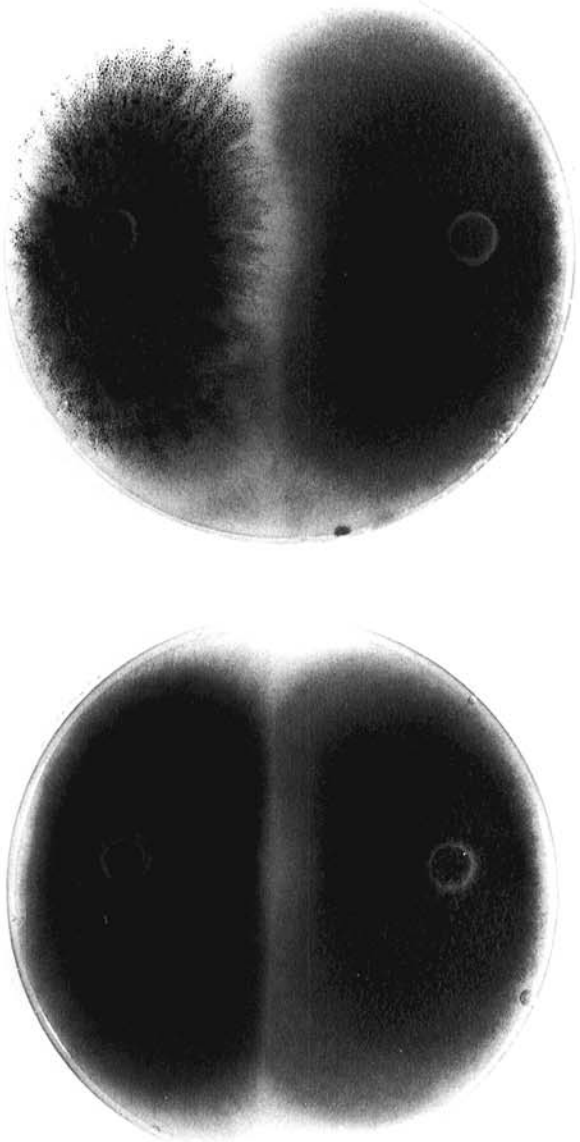

Fig. 2. A, Comparison of original culture, NC1 (left), and culture with altered morphology, NC1-R (right). **B,** Comparison of original culture, BC92 (left), and culture with altered morphology, BC92R (right).

pattern or group were grown on V8 agar for 7 days at room temperature. Subsequently, 7-mm-diameter plugs were taken from the colony margins and transferred onto both fresh V8-agar plates (100 × 15 mm) and into 50 ml of PDB in 100- × 25-mm petri dishes. All agar plates were incubated at 22–23 C, and the PDB plates were placed at room temperature. Radial growth rates were determined from the V8-agar plates by averaging three colony-diameter measurements per plate taken at random intersects at 2- to 3-day intervals.

Total phialospore production was determined after 14 days of growth in PDB (24). The mycelial mat was removed, and the remaining medium was brought to 200 ml with water. The mat was returned to the solution and agitated on a shaker (New Brunswick Scientific, Edison, NJ) for 10 min at 125 rpm. A drop of Tween 80 (polyxyethylene sorbitan monolaurate) was added before shaking to help disperse the spores. The number of phialospores was determined using a hemocytometer and averaging the counts from two to three replicate cultures with 10 readings per culture. Mycelial dry weight also was assessed at this time by collecting the fungal mat onto preweighed Whatman No. 4 filter paper in a Buchner funnel with vacuum filtration. The mats were dried completely at 42 C and weighed. Three replicate plates of V8 agar and two to three dishes of PDB were included for each isolate per experiment, and each experiment was repeated. The data presented are the combined results from two experiments.

Estimation of virulence. Mature carrot (*Daucus carota* L.) roots of different cultivars were obtained from retail stores, surface-sterilized in 0.5% sodium hypochlorite for 5 min, rinsed twice in sterile distilled water, and cut into longitudinal sections (5 cm long × 1 cm wide). The sections were placed in sterile petri dishes lined with moistened Whatman No. 1 filter paper. Two or three mycelial plugs (5 mm in diameter) taken from the same colony were placed (mycelial side down) on the carrot sections at evenly spaced distances. The dishes were incubated at 23 C for 7 days, at which time the extent of colonization of the carrot slice by mycelium was measured at the widest point of growth from each agar plug. For each isolate, there were two replicate dishes, and the experiment was repeated. The data presented are the means from six experiments.

Statistical analysis. The relationship of colony diameter (millimeters) over time (days) was modeled for each isolate, using the logistic equation: $D(t) = M/(1 + Ce^{-\beta t})$ (equation 1), in which M = the maximum plate diameter (84 mm); t = number of days after inoculation; and $C = 11$, i.e., $D(0)$ is the plug diameter of 7 mm. The coefficient β was estimated for each culture by the nonlinear least squares method by the NLIN procedure in SYSTAT (31). The β coefficients, spore counts, dry weight, and virulence data were separately analyzed by the GLM procedure in SAS (28). Plots of residuals were examined to assess variance homogeneity. Data were pooled when variance between experiments was homogeneous and when the interaction between the main factor and experiment was not significant. There was no need for data transformation. The significance of the differences between means was established by the least squares means method (28). The data presented are the combined results from five experiments (for growth rate) or two representative experiments each for sporulation and dry weight.

Curing of dsRNA. Two inhibitors of protein synthesis (i.e., cycloheximide (8) at 0.5, 1.0, and 3.0 μg/ml and emetine (35) at 50, 100, and 500 μg/ml) were added to V8 agar. Mycelial plugs of selected isolates were transferred to these amended media, and the plates were incubated at room temperature in the dark. The plates were examined every few days for visible changes in morphology or growth patterns. If changes were observed, subcultures were made onto fresh V8-agar plates. After 2 wk, DNA/dsRNA extraction was conducted on these variants, as described above, to determine whether changes in dsRNA patterns had occurred. In further studies, phialospore suspensions were diluted and plated onto V8 agar containing 500 μg of emetine per milliliter, and any morphologically distinct colonies were subcultured and assayed, as above. Also, hyphal-tip transfers were made from selected isolates by growing them on 1.5% water agar and cutting agar blocks containing individual hyphae (under the dissecting microscope) and placing them on V-8 agar. These cultures also were examined for dsRNA patterns.

During routine subculturing of the isolates on V8 agar without inhibitors of protein synthesis, one strain (NC1) showed a marked change in morphology by sectoring out (Fig. 1B). Upon subculture

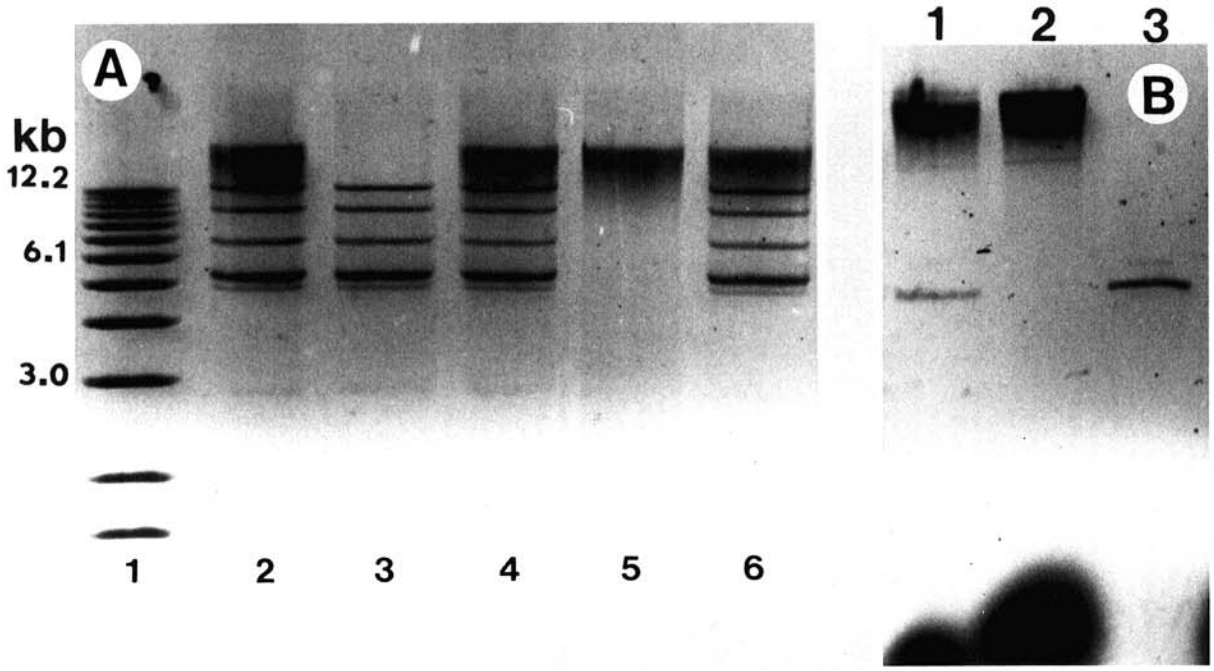

Fig. 3. DsRNA in *Chalara elegans*. **A,** Agarose gel of total nucleic acids from isolate BC92 treated with DNase I or RNase A and stained with ethidium bromide. Lane 1, 1-kb dsDNA ladder; lane 2, sample with no treatment; lane 3, nucleic acids digested with DNase I in 0.04M Tris, pH 7.5, and 0.006 M MgCl; lane 4, nucleic acids digested with RNase A in 0.3 M NaCl; lane 5, nucleic acids digested with RNase A in 0.03 M NaCl; lane 6, nucleic acids incubated in 0.03 M NaCl with no RNase A present. **B,** CF11 cellulose column chromatography of total nucleic acids extracted from isolate CA. Lane 1, total nucleic acids; lane 2, column unbound fraction (DNA and ssRNA); lane 3, column bound fraction (dsRNA).

from this sector, a colony with a distinctively different phenotype was obtained and referred to as an altered phenotype, NC1-R (Fig. 2A). Additional phenotype changes were seen in two other isolates, BC92 and BK28, during the course of this study. The dsRNA patterns in the original and altered strains were determined as described above. In addition, measurements of the extent of mycelial growth (radial and dry weight), sporulation, and virulence were made on NC1-R and BC92-R, as described above.

Virus purification. One isolate (NC-1) was grown in PDB at room temperature for 21 days. The mycelial mats were harvested (total fresh weight = 14 g) and homogenized in phosphate buffer (0.05 M Na/K phosphate, pH 7.0; 5 ml/g) containing 0.1% mercaptoethanol with a blender at high speed for 30 s. This was followed by further grinding in a hand-held homogenizer with a B pestle and shaking for 1 h at 4 C. The homogenate was clarified by centrifugation at 6,000 *g* for 15 min (low-speed centrifugation). Sodium chloride (0.6 M) and polyethylene glycol (PEG, 15%) were added slowly to the resulting supernatant and stirred for 1 h at 4 C. The precipitate that formed was collected by low-speed centrifugation and resuspended in 0.05 M Na/K phosphate buffer (pH 7.0). The solution was gently agitated overnight at 4 C. The suspension was clarified by low-speed centrifugation and then subjected to a high-speed spin (Ti50-2 rotor, Beckman Instruments, Inc., Fullerton, CA; 102,000 *g*). The resulting pellets were resuspended in 300 μl of 0.05 M Na/K phosphate with gentle shaking overnight at 4 C. The next day, the solution was overlaid onto a 10–40% sucrose gradient buffered with 0.05 M Na/K phosphate. The gradients were centrifuged in a Beckman SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) at 38,000 rpm for 2 h at 4 C and scanned with an absorbance monitor at 254 nm. Fractions showing maximum UV absorbance were pooled and dialyzed against phosphate buffer. Purified particles were prepared for transmission electron microscopy.

TABLE 2. Double-stranded RNA (dsRNA) in isolates of *Chalara elegans* showing sizes of main fragments after electrophoresis in 0.7% agarose[a]

Fragment sizes (kb)	Isolates	Group designation
2.8	BCA; BC92CO; BK: 16-1, 18, 25; CA1; HA; WASH; BC92-R[b]; NC1-R	A
5.3, 2.7	BK: 16-2, 34-2	A-1
5.3, 5.0, 3.6, 2.6[c]	AK: 4N1, 89-2 89-3, 89-4, 89-7, 308; BK28; F374; TX; ATCC: 9853, 34114; CKP B	
6.6, 5.3, 5.0, 3.6, 2.6[c]	BK101; OR1, OR2; ATCC18722; HRB	B-1
12, 6.8, 5.3, 5.0, 3.6, 2.8[d]	NC: 1, 2, 1526	C
12, 6.8, 6.0, 5.3	NC: 30, 49	C-1
12, 7.4, 6.6, 5.3, 4.9	ATCC9109	C-2
12, 6.0, 5.3, 4.9, 3.0, 2.8[d]	NC: 1527, 17	C-3
12, 9.2, 6.8, 5.3, 5.0, 2.8[d]	BC92	D
no dsRNA	AK: 89-1, 89-6, 208; BK: 102, 103, 38-1; FL-W; BK28-R	E

[a] Fragment sizes are ±100 bp.
[b] The letter R denotes a culture with altered morphology.
[c] The bands ranging from 2.6 to 3.6 kb were sometimes absent.
[d] A band at 2.8 kb was sometimes absent.

RESULTS

Characterization of dsRNA in *C. elegans*. DsRNAs were detected in 36 of the 43 strains of *C. elegans* examined in this study that originated from widely different geographic areas and substrates, including four from ATCC. The dsRNA nature was confirmed by insensitivity to both DNase and RNase at high ionic strength and sensitivity to RNase at low ionic strength (Fig. 3A). In addition, dsRNA segments were eluted from a CF11 cellulose column that concentrates dsRNA (Fig. 3B). All of the *C. elegans* isolates that contained dsRNAs were assigned to nine groups based on their general similarities in the banding pattern of the dsRNA fragments after electrophoresis on 0.7% agarose gels. The sizes of the dsRNA fragments observed within the respective isolates are given in Table 2. The dsRNA patterns for 30 of the 36 isolates are shown in Figure 4. Isolates representing groups A, B, C, and D also were compared directly with one another (Fig. 5A).

Hybridization experiments were conducted at both high and low stringency with a probe constructed from the 2.8-kb dsRNA fragment in isolate CA1 from group A. At high stringency, the probe hybridized with the 2.8-kb band from eight other tested isolates in group A, not including isolate CA1. No cross-hybridization was observed with the higher molecular weight dsRNA fragments present in isolates in groups B, C, or D (Fig. 5B). However, cross-hybridization was observed with these fragments when the probe was tested at low stringency (*data not shown*).

Morphology, growth, sporulation, and virulence. The morphology of *C. elegans* isolates on V8 agar varied from uniform light to dark brown in color (most common to group A and D isolates) to a dark-brown and black pigmentation with serrated colony edges and the presence of exudate droplets (exemplified by groups B and C) (Fig. 1A). The isolates that contained no dsRNA had a wide range of morphologies. The colony diameter of isolates with dsRNA patterns representative of groups A–E was recorded every 2–3 days over a 20-day period at 23 C. For each isolate, there was a characteristic logistic growth curve (Fig. 6). The adjusted R^2 value resulting from individual statistical analysis was 0.98 or greater for all cases, indicating that the chosen logistic

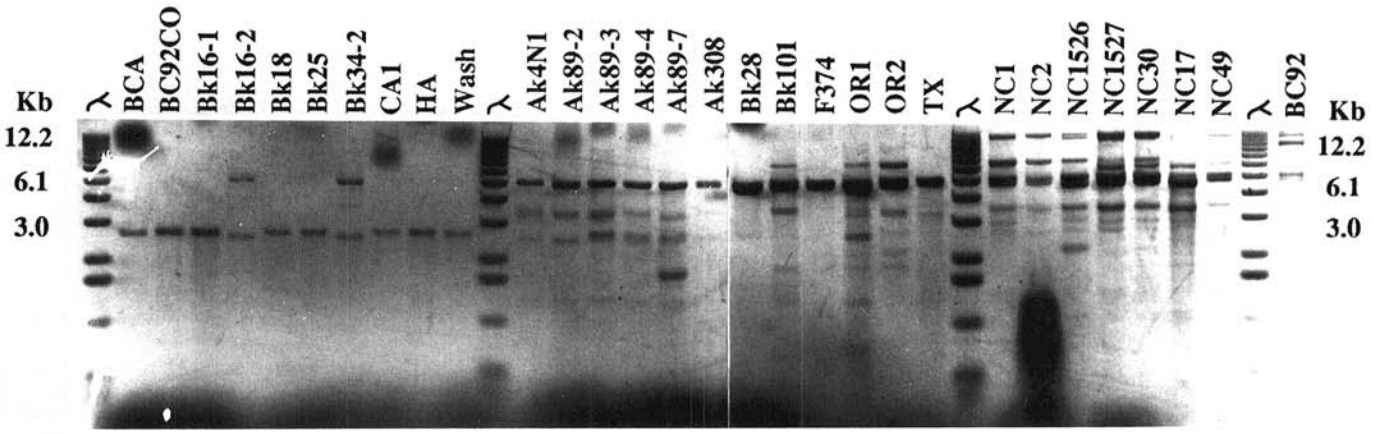

Fig. 4. DsRNA banding patterns in 30 isolates of *Chalara elegans*. Samples were electrophoresced in 0.7% agarose gels and stained with ethidium bromide.

model was appropriate. Five growth-rate experiments were analyzed by statistical methods. The variance between the experiments appeared homogenous, and the interaction between treatment and experiment was not significant; therefore, the overall analysis was conducted with all the combined data. Significant differences ($P = 0.008$) in the growth rates were found. Group E (no dsRNA) grew at a significantly ($P = 0.05$) faster rate, whereas group C (multiple dsRNAs) grew significantly slower in comparison with the other groups. Groups A, B, and D were not significantly different from each other (Fig. 7A).

No significant differences in mycelial dry weight ($P = 0.10$) and sporulation ($P = 0.31$) were apparent between isolates in each of the four selected dsRNA-containing groups (Fig. 7B and C), possibly due to large variations between isolates. Representative isolates from each of the five selected groups also were compared for the extent of colonization of carrot-root slices. The results showed that group E (no dsRNA) isolates were significantly less virulent ($P = 0.01$) than were the other isolates, whereas group A isolates proved to be the most virulent (Fig. 7D).

Curing of dsRNA. During subculturing on V8 agar, isolate NC-1 (group C) developed a morphologically distinct sector when compared to its original morphology (Fig. 1B). This sector yielded a culture (NC1-R) that was similar morphologically to isolates in group A. Nucleic acid extraction revealed that none of the high molecular weight dsRNA bands were present any longer, whereas the intensity of the 2.8-kb band was enhanced (Fig. 8A). This latter band also was subsequently detected in the high-stringency hybridization experiments using the 2.8-kb fragment from group A as a probe, indicating there was some homology (Fig. 5B).

Representative isolates containing various dsRNA bands were grown on V8 agar amended with either cycloheximide HCl or emetine. At all of the concentrations tested, cycloheximide was inhibitory to growth of the fungus. During this experiment, a change was observed in one isolate (BC92) in group D from a darkly pigmented colony color to a light-brown color (Fig. 2B). Nucleic acid extraction from this culture with altered morphology (BC92-R) indicated that the high molecular weight bands were no longer present, and there was increased intensity in the 2.8-kb band (Fig. 8A). Hybridization of this band with the 2.8-kb probe, as noted for NC1-R, also was observed (*data not shown*). Attempts to eliminate the dsRNA by obtaining single-phialospore cultures on V8 agar supplemented with emetine or by hyphal tipping were not successful, and the dsRNA patterns were not altered.

Mycelial growth, sporulation, and virulence of partially cured strains. Analyses of data were conducted on the isolates with altered morphology and dsRNA patterns from groups C and D, described above (NC1-R and BC92-R). There was a significant ($P = 0.03$) increase in growth rate in NC1-R compared to the original NC-1 isolate (Figs. 6 and 9A). This was not, however, seen for BC92-R ($P = 0.32$). When spore counts and dry weight of NC1 and NC1-R were examined, mycelial weight was 25% higher in NC1-R ($P = 0.054$). Spore counts in NC1-R were consistently lower from experiment to experiment, but the significance level ($P = 0.15$) was not acceptable. Because the group D isolate (BC92-R) was tested only once, data are not reported. A significant ($P = 0.003$) increase in virulence was seen in NC1-R compared to NC-1 (Figs. 8B and 9B). This trend was seen in seven repeated experiments, permitting an analysis with all the combined data. The isolate BC92-R also had increased virulence ($P = 0.06$) (Figs. 8C and 9B).

Virus purification. After extraction and purification from strain NC-1 (group C), isometric VLPs with a mean external diameter of about 47 nm (SD ± 2.3 nm, $n = 14$) were observed (Fig. 10). A few bacilliform virions (approximately 120 × 30 nm) also were observed.

DISCUSSION

C. elegans is a widespread pathogen that causes black root rot and seedling damping-off on a number of plant species (10,17–19,26). In previous pathogenicity studies, differences in virulence among isolates of *C. elegans* were noted (15,17,32). Similarly, isolates differ in morphology when grown in culture (15). However, the basis for these differences has not been examined. In some fungi, the presence of dsRNA is associated with changes in fungal virulence and morphology (1,3,5,12,33). There is only one previous report of the occurrence of dsRNA in *C. elegans* (4). In the present work, dsRNA fragments were detected

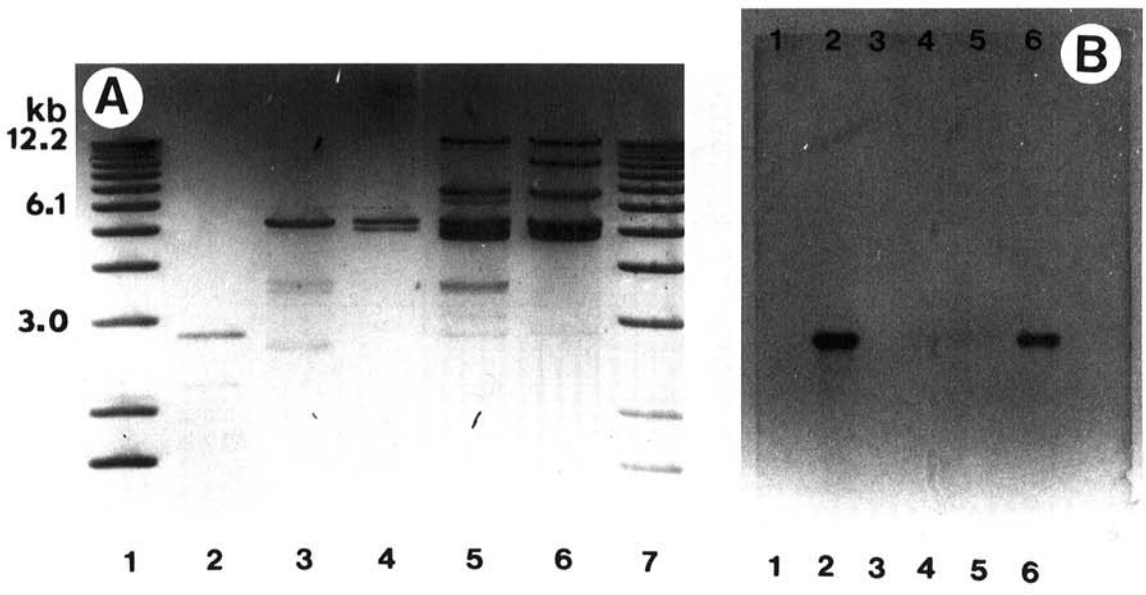

Fig. 5. A, Agarose gel (0.7%) electrophoresis of dsRNA extracted from isolates with dsRNA banding patterns representative of groups A, B, C, and D, stained with ethidium bromide. Lane 1, 1-kb dsDNA ladder; lane 2, dsRNA from BCA (group A); lane 3, dsRNA from AK4N1 (group B); lane 4, dsRNA from BK28 (group B, 3.6- and 2.6-kb bands absent); lane 5, dsRNA from NC2 (group C); lane 6, dsRNA from BC92 (group D); lane 7, 1-kb dsDNA ladder. **B,** Hybridization of 2.8-kb dsRNA from isolate CA (group A) to dsRNA of other isolates. The dsRNAs from various isolates representing the different dsRNA banding pattern groups were run in 1.0% agarose gel and blotted onto Gene bind nylon membrane (Pharmacia). A cDNA probe was labeled with nonradioactive digoxigenin-labeled dUTP by reverse transcriptase. Lane 1, 1-kb dsDNA ladder; lane 2, isolate BK25 (group A); lane 3, isolate BK28 (group B); lane 4, isolate BC92 (group D); lane 5, isolate NC1 (group C); lane 6, NC1-R (group A).

in 36 of 43 isolates of *C. elegans* studied. Because these isolates displayed a wide range of morphologies, further investigation into possible effects of dsRNA was pursued.

The dsRNA patterns observed after agarose gel electrophoresis generally were consistent even after extensive subculturing and storage of the isolates over a 24-mo period (A. M. Bottacin, C. A. Lévesque, and Z. K. Punja, *unpublished observations*). This stability allowed the placement of isolates in nine groups based on dsRNA patterns, with a tenth group containing no dsRNA. The groupings were made for categorizing isolates and do not necessarily imply that the dsRNAs were similar or related. There appeared to be no consistent correlation between observed differences in morphology and these dsRNA patterns, with the exception of the group C isolates, in which the morphology was very distinctive and all eight isolates had unique, multiple (four to six bands) dsRNA patterns. Isolates in group B exhibited the most variation with respect to morphology, and they also contained several combinations of faint dsRNA bands. Despite having some dsRNA bands in common with group C, the same consistent correlation between morphology and dsRNA patterns could not be obtained for the isolates in group B. These isolates originated from several geographic areas and substrates; in contrast, group C isolates were all from the same geographic area (North Carolina) and substrate (tobacco). Therefore, it is likely that factors other than the presence of dsRNA are responsible for the differences in morphology observed within group B isolates. The dsRNA pattern observed in the group D isolate (BC92) also was very

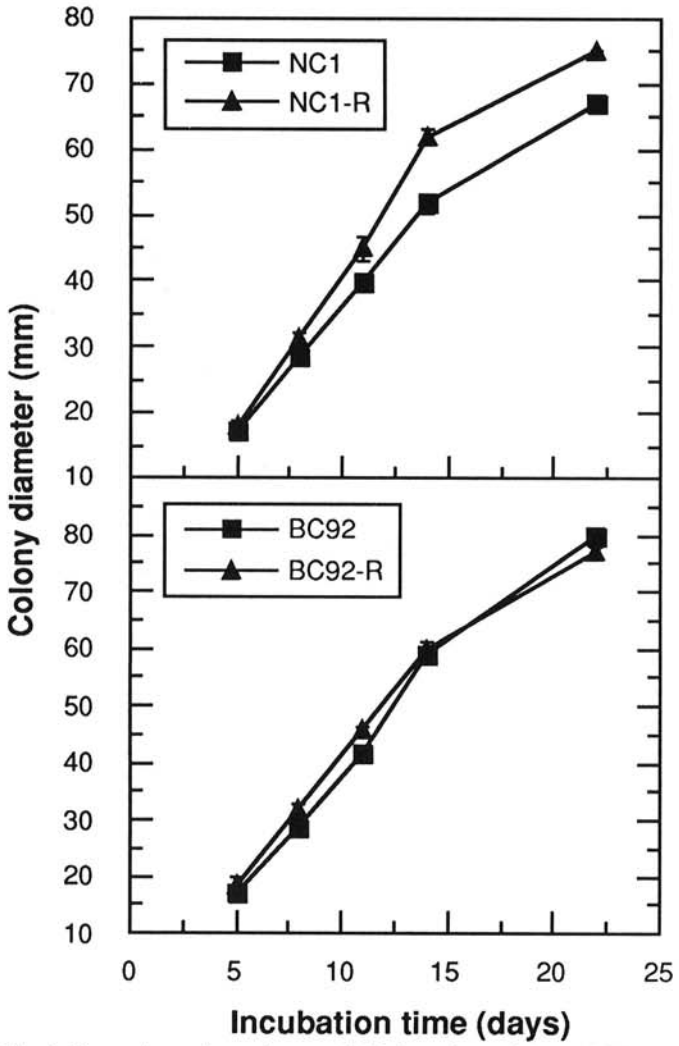

Fig. 6. Comparison of growth rate of *Chalara elegans* isolates NC1 and BC92 with NC1-R and BC92-R. **A,** NC1 versus NC1-R. **B,** BC92 versus BC92-R. Cultures were grown on V8 agar at 22–23 C; measurements were made every 2–3 days from three replicate dishes.

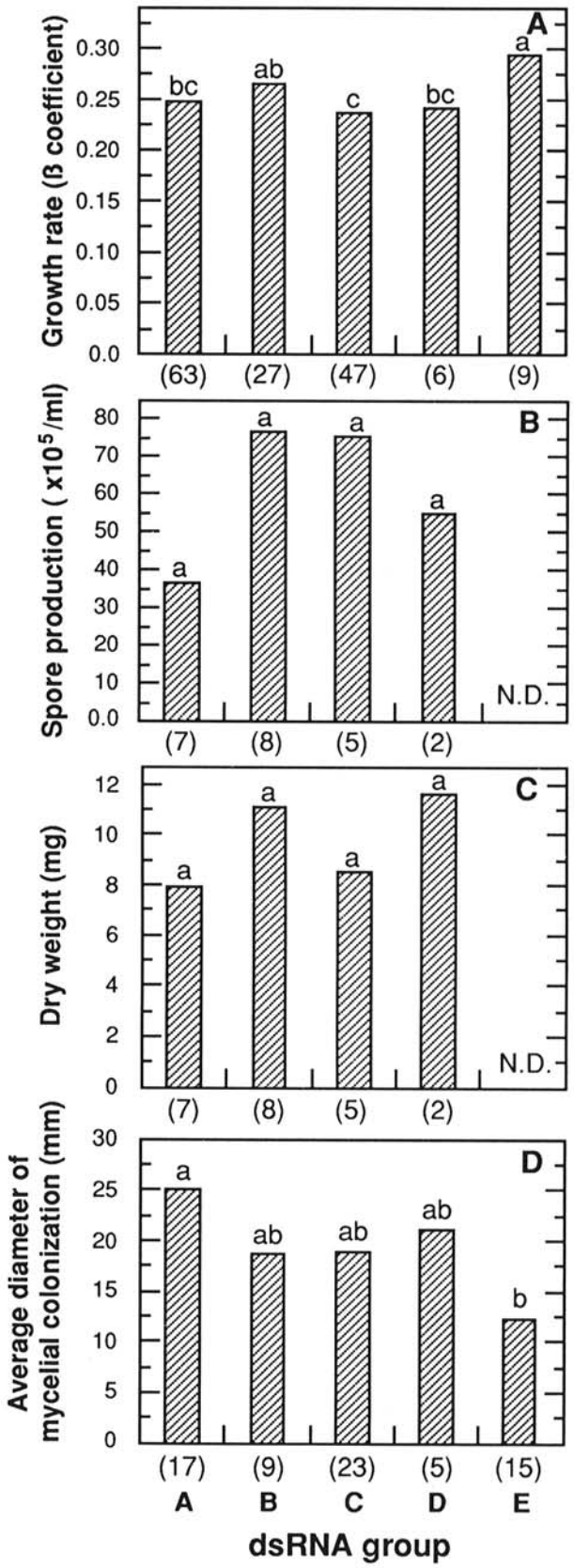

Fig. 7. Comparison of physiological parameters in isolates of *Chalara elegans* containing different dsRNA fragments. **A,** Growth rate, average of five experiments. **B,** Spore production, average of two experiments. **C,** Dry weight, average of two experiments. **D,** Virulence, average of seven experiments. Columns with the same letter are not significantly different ($P < 0.05$) as determined by difference of least squares means. Numbers in parentheses below columns indicate total number of cultures used in the analysis.

similar to that of the group C pattern, but the morphology was different. In BC92, there was a unique dsRNA segment (9.2 kb) not present in group C isolates that may account for the difference.

The ATCC cultures used in this study provided additional information about the widespread occurrence of dsRNA within *C. elegans*. ATCC9853 originated from New Zealand and, thus, represented a distant geographic region. In addition, ATCC18722 has been in culture since June 1969, indicating the stability of dsRNA within *C. elegans*. The banding pattern of ATCC9853 and ATCC34114 placed them in group B, whereas ATCC18722 was placed in group B-1. The dsRNAs of ATCC9109 were within the molecular size range observed in other isolates, but the pattern was unique, placing it in a different subgroup.

Measurements of growth rate, sporulation, dry weight production, and virulence, using representative cultures from each of four dsRNA-containing groups, indicated that only isolates in group C were different from the other isolates in the majority of the experiments. Although the trends were clearly toward reductions in growth rate, dry weight production, and virulence, and an increase in phialospore production, a statistically significant difference could be shown only for growth rate and virulence. In the case of dry weight and sporulation data, large variations among the isolates tested masked any differences. It should be noted that in three of the nine experiments, the trend for group C was not supported, because growth rate was not reduced, phialospore production was normal, and morphology was not as extreme. In *Lentinula edodes*, some strains that harbored dsRNA also did not exhibit any of the characteristic symptoms associated with dsRNA infection (27). This was attributed to a "latent residential virus" condition, and a similar phenomenon could be occurring in *C. elegans*. Variation also was observed in some of the virulence studies, such that the dsRNA pattern was not always correlated with differences in virulence. Similar discrepancies were noted in earlier studies of *Endothia parasitica* strains containing dsRNA (1). Therefore, although some of the data appear to support a direct influence of dsRNA on selected fungal physiological parameters, they only provide putative correlative evidence because the genetic background of the isolates studied was different. Elimination of the dsRNA within

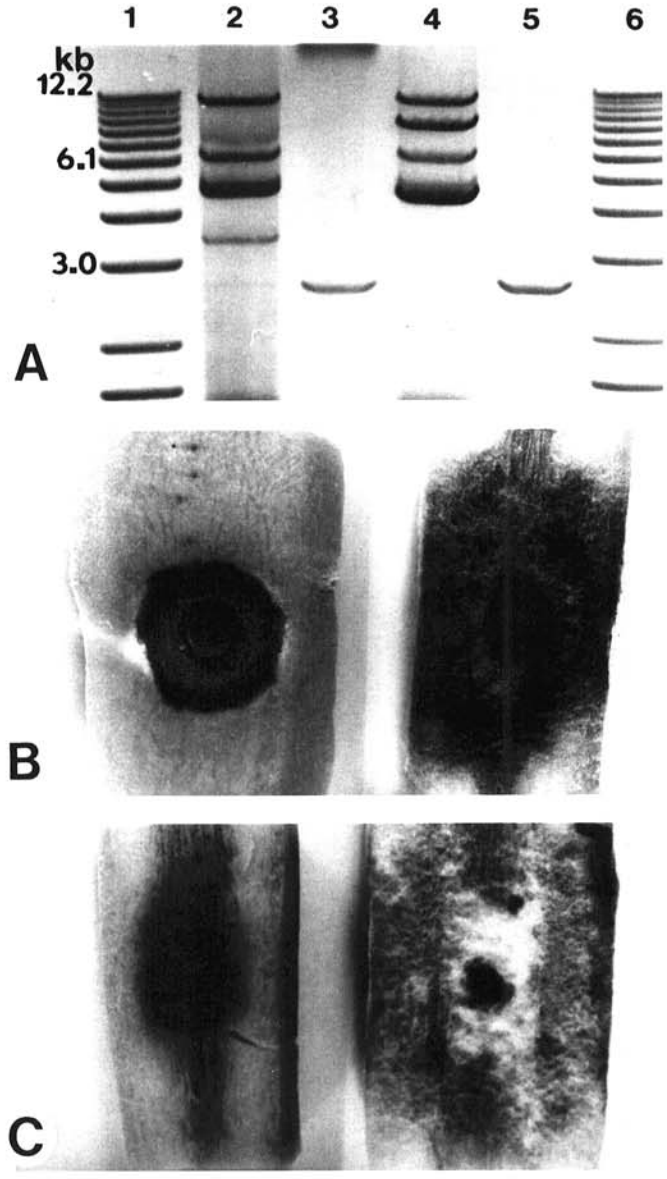

Fig. 8. DsRNA and virulence of revertant cultures of *Chalara elegans*. **A,** Agarose gel electrophoresis of dsRNA extracted from isolates NC1 and BC92 and those with altered morphology, NC1-R and BC92-R. Lane 1, dsDNA ladder; lane 2, dsRNA pattern of NC1; lane 3, dsRNA pattern of NC1R; lane 4, dsRNA pattern of BC92; lane 5, dsRNA pattern of BC92R; lane 6, 1-kb dsDNA ladder. **B,** Virulence test on carrot-root slices for isolates NC1 (left) and NC1-R (right). **C,** Virulence test on carrot-root slices for isolates BC92 (left) and BC92-R (right).

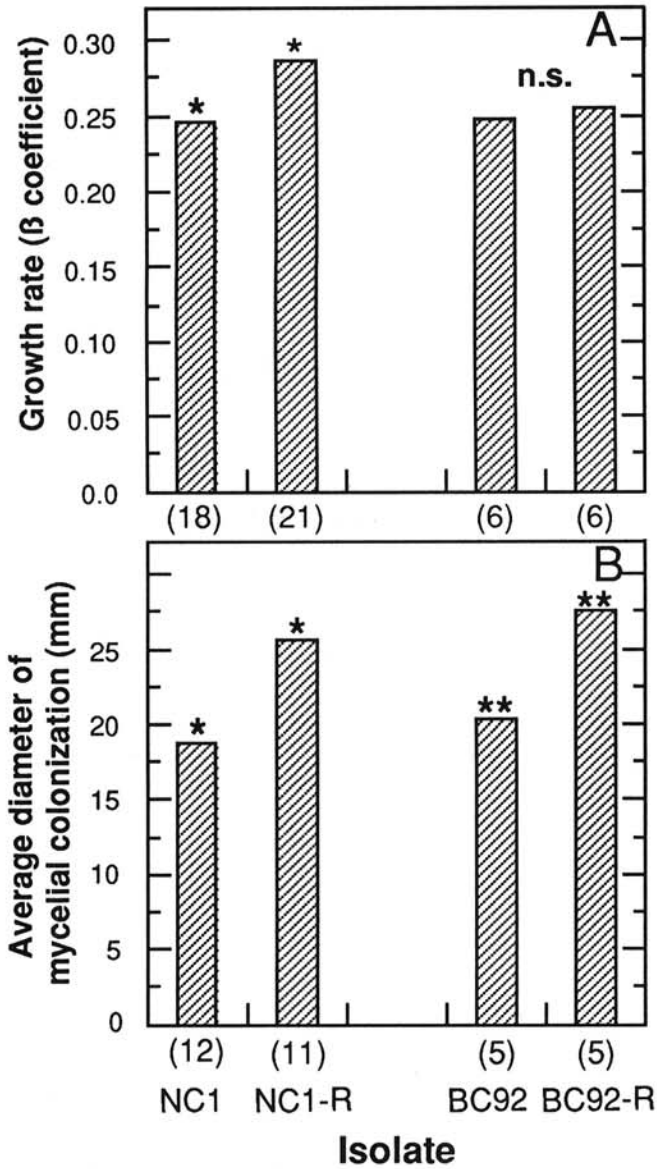

Fig. 9. Comparison of physiological parameters in *Chalara elegans* isolates NC1 and BC92 with NC1-R and BC92-R. **A,** Growth rate. **B,** Virulence. Asterisks over columns indicate significant difference (* is $P < 0.05$ and ** is $P = 0.06$) between pairs. Numbers in parentheses below columns indicate total number of cultures used in the analysis.

the same genetic background would provide more convincing evidence for the effects of dsRNA in *C. elegans*. This was in fact achieved in this study by the detection of three cultures with altered morphology originating from groups B, C, and D.

For the isolate in group C (NC1), spontaneous elimination of five high molecular weight dsRNA segments and a corresponding increase in the intensity of a 2.8-kb segment resulted in a dramatic change in morphology to that resembling group A isolates. In addition, an increase in both growth rate and virulence to a level comparable to that for isolates in group A was seen. In the isolate from group D (BC92), the similar loss of four multiple dsRNA segments and the appearance of a 2.8-kb band resulted in an increase in virulence ($P = 0.06$) and a change in colony color but not in growth rate. Originally, this altered morphology was thought to result from the presence of emetine in the medium, but it now appears to have been a random and rare event. In both of the isolates with altered morphology, the 2.8-kb fragment was retained. A third culture with altered morphology (BK28-R) that was recovered from group B had all dsRNA eliminated. The result of this change was a more normal morphology (i.e., similar to group A) and an increase in growth rate, but there was a noticeable reduction in virulence (*data not shown*). Isolates in group E (no dsRNA) also appeared to have reduced virulence. It is conceivable that the absence of the 2.8-kb fragment, or all dsRNAs, in isolates of *C. elegans* may reduce virulence, but this requires further study. Although the presence of dsRNA generally is believed to attenuate virulence, it has been suggested that the exceptional virulence as well as the diversity of virulence in the Mexican isolates of *Phytophthora infestans* may be due to factors encoded by the dsRNAs found in those strains (21).

A 2.8-kb band was occasionally seen in the original group C and D isolates, but it was faint in comparison to the other bands. Therefore, the enhancement of the 2.8-kb band in cultures with altered morphology from these groups may be due to the release of inhibition or greater access to necessary dsRNA replication factors that previously may have been sequestered by the multiple and larger dsRNA segments in the original culture. The presence of diverse dsRNAs in *Cryphonectria parasitica* was proposed to be due to a dynamic competition between defective and genomic hypovirulence-associated dsRNAs that could have a visible effect on phenotypic expression (29). The effect of the interaction between different dsRNAs in *C. elegans* is unknown and deserves further attention. In addition, a comparison of sequence similarities should provide interesting insights into the origins and relationships of these dsRNAs in this plant pathogen.

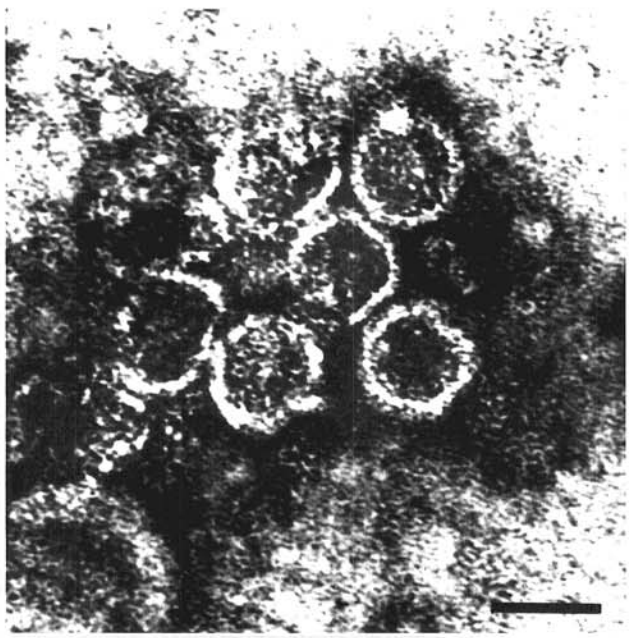

Fig. 10. Electromicrograph of virus-like particles purified from *Chalara elegans* isolate NC1. Bar = 50 nm.

To test the degree of homology between the different dsRNA fragments, hybridization studies were conducted with the 2.8-kb segment as a probe template. At high stringency, no cross-hybridization was observed with the dsRNAs of higher molecular weights, whereas the 2.8-kb segment in isolates in group A and in NC1-R and BC92-R showed homology. Thus, despite the different geographic origins of these isolates, they all appeared to contain a putatively similar dsRNA fragment.

VLPs were partially purified by a procedure involving PEG-NaCl precipitation, differential centrifugation, and equilibrium centrifugation. Preliminary electron microscopy studies revealed the presence of isometric particles approximately 47 nm in size in isolate NC-1 (group C). Bozarth and Goenaga (4) reported the occurrence of isometric particles approximately 40 nm in diameter in *T. basicola* (synanamorph *C. elegans*), and electrophoresis of the nucleic acids extracted from this VLP preparation yielded five bands that ranged in molecular weight from 2.7 to 4.5×10^6 Da after electrophoresis on a polyacrylamide gel. It was suggested that the VLPs in *T. basicola* represented a complex mixture of five viruses, each with one dsRNA segment (4,5). Four of the six dsRNA bands in isolates in group C may have molecular weights comparable to those reported by Bozarth and Goenaga (4). We examined the original culture (ATCC34114) by agarose gel electorphoresis and found two major bands at 5.3 and 4.8 kb, with three faint bands at 3.8, 2.7, and 1.9 kb, tentatively placing the isolate of Bozarth and Goenaga (4) in our group B (*data not shown*).

Evidence continues to emerge that supports the theory that the persistence of cytoplasmic genetic elements within the fungal host involves a transfer of information between the nuclear and mitochondrial genes of the fungus and the dsRNA genome (22,36). It has been shown that an altered fungal phenotype (reduced laccase production) is the consequence of a specific action by the dsRNA on fungal gene expression in *Cryphonectria parasitica* and not a general response (6). Recent studies have shown the feasibility of transferring hypovirulence-associated traits to an isogenic virulent strain of *Cryphonectria parasitica* (7). Furthermore, analysis of the largest dsRNA segment in *Cryphonectria parasitica* revealed a structural resemblance with plus-strand RNA virus genomes. Five distinct domains were strikingly similar to the conserved domains within plant potyvirus-encoded polyproteins (16). These recent developments are exciting and encourage further investigation into the role of transcribed sequences of the dsRNA in *C. elegans* (a wide host range facultative parasite) and other plant pathogenic fungi.

Attempts to transfer the dsRNA to strains of *C. elegans* lacking dsRNA have been unsuccessful due to a lack of hyphal anastomosis (A. M. Bottacin, C. A. Lévesque, and Z. K. Punja, *unpublished data*). The transfer of multiple dsRNA fragments and a corresponding reduction in growth rate, virulence, and changes in morphology would provide additional evidence to substantiate our conclusions based on the spontaneous cultures with altered morphology described in this study. Our results indicate that multiple dsRNAs are common within *C. elegans* and can reduce growth and virulence of the pathogen. Research on host-pathogen interactions and pathogenicity should consider the widespread occurrence of dsRNA in *C. elegans* and the possibility that the results may be affected.

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