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Two Nonhomologus Viruses of *Cryphonectria (Endothia) parasitica* Reduce Accumulation of Specific Virulence-Associated Polypeptides†

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Double-stranded RNA responsible for transmissible hypovirulence of *Cryphonectria (Endothia) parasitica* affected the accumulation of specific polypeptides. Nonhomologous hypovirulence-causing double-stranded RNAs, originating in Europe or North America, affected accumulation of the same polypeptides. Fewer than 5% of detectable proteins were affected, indicating that hypovirulence is probably not the result of general debilitation of the fungus.

Cryphonectria (Endothia) parasitica (Murr.) Barr. is a fungal plant pathogen whose virulence can be affected by the acquisition of double-stranded RNA (dsRNA) viruses (2, 9). Hybridization studies of the dsRNA viruses have indicated that there are at least four homology groups (5; C. P. Paul and D. W. Fulbright, Phytopathology, p. 1325, 1985), two of which we examine here. Cultural phenotypes of hypovirulent strains of the fungus are a function of the dsRNA present but generally include reduced numbers of pycnidia, changes in colony morphology, and with some dsRNA types, reduced pigmentation (1). The dsRNAs do not have protein coats but are packaged within fungal vesicles (3). The discovery of differential accumulation of poly(A)$^+$ RNA (7) in virulent and hypovirulent cells prompted this study of differential accumulation of polypeptides.

The virulent strains used were EP155/2 and Gramma 13-10-3. The hypovirulent strains used were UEP1, EP113, and EP915. Strains EP155/2 and UEP1 were isogenic (7). The strains were grown under identical conditions in EP liquid medium (3) at 25°C for 8 days. The mycelium was collected and lyophilized as previously described (7). The mycelium was ground to a fine powder, and 0.1 g was suspended in 2.0 ml of 1.0% sodium dodecyl sulfate–5.0% 2-mercaptoethanol–0.01 M Tris hydrochloride (pH 6.8) and homogenized. The sample was heated to 95°C for 5 min and then centrifuged at 11,500 × *g* for 1 min. The supernatant was diluted with sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2.0% sodium dodecyl sulfate, 0.5% 2-mercaptoethanol, 20% glycerol) and run on a 12.5% Laemmli-type gel (4).

The virulent strains EP155/2 and Gramma 13-10-3 yielded a major protein band with a relative molecular mass of approximately 24 kilodaltons (kDa) (Fig. 1). The hypovirulent strains UEP1, EP113, and EP915 did not contain this band. This procedure was repeated three times and yielded the same results. Strains UEP1 and EP113, which contain a European-type dsRNA, gave the same results as EP915, which contains an American-type dsRNA. Preliminary examination of culture filtrates of these strains grown in EP minimal media (8) also showed a differential accumulation of specific polypeptides. One of these polypeptides was approximately the same size as the 24-kDa protein shown in Fig. 1.

For two-dimensional gel electrophoresis (6), total soluble proteins were extracted by suspending 0.1 g of ground mycelium in 1.0 ml of lysis buffer A (4% Nonidet P-40, 50

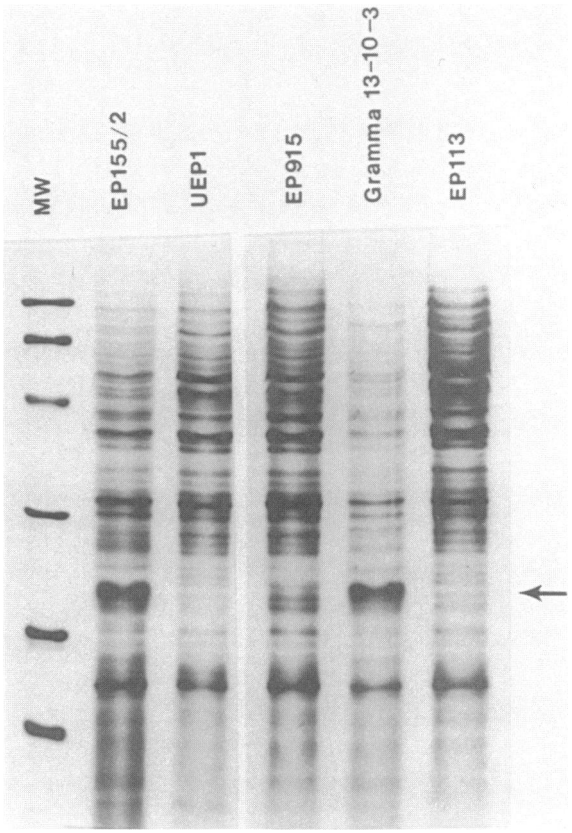

FIG. 1. Single-dimensional protein gel. Total soluble proteins were extracted from virulent and hypovirulent strains and subjected to electrophoresis through a 15% polyacrylamide gel. Each sample was extracted from an equal dry weight of mycelium. The arrow points to the band which appears in the virulent strains but not in the hypovirulent strains. The relative molecular masses of the markers, from top to bottom, are 92.5, 66.2, 45, 31, 21.5, and 14.4 kDa.

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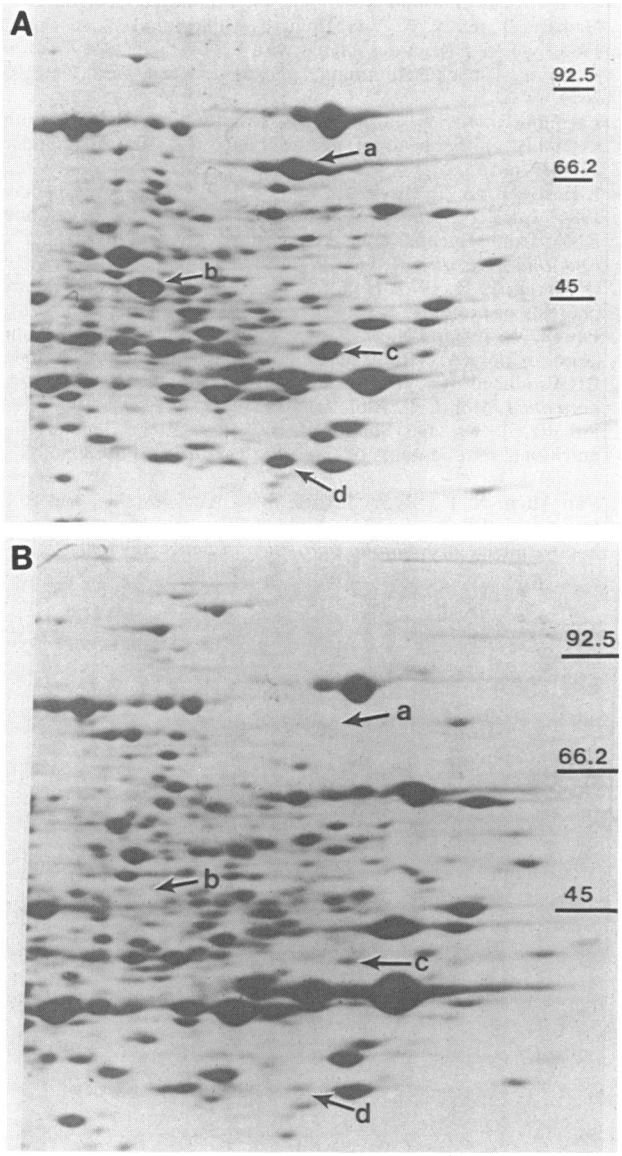

FIG. 2. Upper portions of two-dimensional protein gels. Enlargement of two duplicate 12.5% polyacrylamide gels showing protein patterns of EP155/2 (A) and UEP1 (B). Each gel represents proteins from 20 mg (dry weight) of mycelium. The arrows point to the differentially accumulated proteins. Approximate positions of molecular mass markers are shown to the right.

mM $MgCl_2$, 50 μg of RNase A [Sigma Chemical Co., St. Louis, Mo.] per ml, 100 μg of DNase [Sigma] per ml, 1.0% 3/10 ampholytes, 2% 6/8 ampholytes, and 2% 4/6 ampholytes [Bio-Rad Laboratories, Richmond, Calif.]) and homogenized. An equal volume of lysis buffer B (4% Nonidet P-40, 10% 2-mercaptoethanol, 9.0 M ultrapure urea) was added, and the mixture was homogenized again. The samples were centrifuged at 11,500 × *g* for 3 min, and various dilutions of the supernatant were loaded onto the gels. The equivalent of 2.0 mg of lyophilized mycelium appeared to be optimal for electrophoresis. Gels used for the second dimension of electrophoresis were 12.5% acrylamide–2.7% *N,N'*-methylene-bis-acrylamide (Bio-Rad). The gels were stained with Coomassie blue R-250.

Results of two-dimensional gel electrophoresis are shown in Fig. 2 and 3. Eight predominant protein spots appeared in gels of the virulent form EP155/2 of the two isogenic strains but were absent or much reduced in the hypovirulent form UEP1. The relative molecular masses of these spots were approximately 66, 50.0, 44.5, 35.5, 31, 30, 28, and 27 kDa. These polypeptides represent the most easily detectable protein spots that consistently showed strain differences from gel to gel.

Another virulent strain, Gramma 13-10-3, contained all eight proteins, as did EP155/2. The hypovirulent strains EP113 and EP915 were similar to UEP1 in that they either lacked these proteins or showed much-reduced concentrations. The dsRNAs in EP915 are not homologous to those in UEP1 or EP113, yet the dsRNAs had the same effect on the accumulation of these proteins.

These results showed that dsRNA-induced hypovirulence causes a differential accumulation of specific polypeptides. These polypeptides constitute less than 5% of detectable proteins. It appears, therefore, that hypovirulence is associated with a perturbation of specific gene expression. This hypothesis is supported by previous research in which we showed a differential accumulation of two specific mRNAs in virulent but not hypovirulent strains (7). One of these

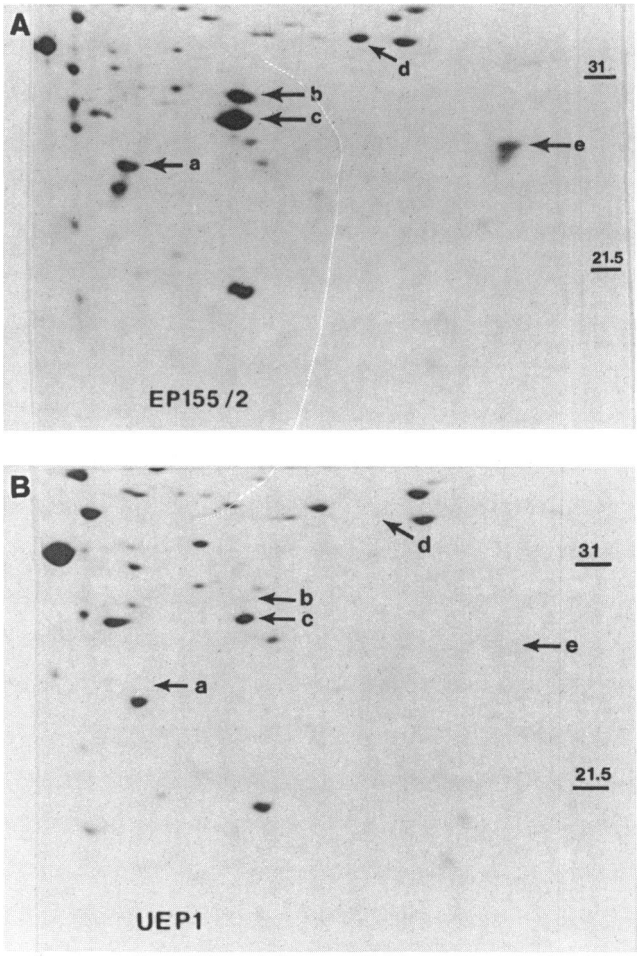

FIG. 3. Lower portions of the two-dimensional protein gels partially shown in Fig. 2. Protein d is the protein d in Fig. 2, used to show the overlap of the figures. Arrows point to differentially accumulated proteins.

mRNAs is 0.65 kilobases, a size which would encode a 24-kDa protein.

The functions of these proteins are unknown. Some of them probably are involved in virulence expression, since they are found only in virulent strains. At least one of these polypeptides (24 kDa) is found in the culture filtrate. The cultures from which the mycelium was harvested contained no detectable spores or sporulating structures. It is, therefore, unlikely that these are sporulation-specific proteins. Isolation and characterization of these proteins have begun and may elucidate the mechanisms of virulence and hypovirulence of *C. parasitica*.

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