Lack of Sequence Homology between Double-stranded RNA from European and American Hypovirulent Strains of *Endothia parasitica*

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**SUMMARY**

Dot blots of dsRNA from European and American hypovirulent (H) strains of the chestnut blight fungus, *Endothia parasitica*, were hybridized with $^{32}$P-5'-end-labelled fragments of denatured dsRNA of French, Italian and American origins. Although dsRNA from European or American strains reacted well with labelled RNA probes from strains from the same continent there was little or no cross-hybridization between RNA from strains from different continents.

Hypovirulence in the chestnut blight fungus *Endothia parasitica* (Murr.) And. is cytoplasmically controlled. Transmission of cytoplasmic determinants from hypovirulent (H) strains to compatible normal virulent (V) strains following hyphal anastomosis, in the host or in culture, is responsible for conversion of V strains to H forms (Grente & Sauret, 1969a, b; Van Alfen *et al.*, 1975). Present evidence indicates that dsRNAs, presumably of viral origin, are the cytoplasmic determinants for hypovirulence (Day *et al.*, 1977; Day & Dodds, 1979).

The dsRNAs occur in many size classes in individual H strains (Dodds, 1980a). Thus, for an understanding of the role of dsRNA in hypovirulence, it is important to determine the relatedness of dsRNA components within a given H strain and between those of different H strains. Based on electrophoretic banding patterns, Dodds (1980a) proposed to classify dsRNAs into three types; types I and II encompass the European H strains, and type III comprises all H strains found naturally in the United States. Unfortunately, electrophoretic banding patterns cannot be relied upon for determining the interrelationships among dsRNAs from different sources since variations in number, size and relative abundance of the electrophoretic components were observed upon repeated subculturing of H strains or transfer of dsRNA to a new strain (Dodds, 1980b; Anagnostakis & Day, 1979; Anagnostakis, 1981; Van Alfen, 1982).

The present study examines the relatedness of dsRNA from European and American H strains using dot blot hybridization.

A set of eight strains of *E. parasitica* (supplied by the Connecticut Agricultural Experiment Station) was used comprising an American V strain (EP 155) and seven H strains derived from it. The H strains were obtained by pairing EP 155 on the surface of cellophane over nutrient agar medium with each of seven different H strains of Italian, French and American origins (Anagnostakis, 1981; Elliston, 1982). The original H strains, their country of origin, and the H strains resulting from the conversion of EP 155 are listed in Table 1.

Cultures of all strains were maintained on potato dextrose agar supplemented with DL-methionine (100 mg/l) and biotin (1 mg/ml) (Anagnostakis & Day, 1979). Mycelia used for nucleic acid extraction were grown in stationary cultures on potato dextrose broth supplemented with methionine and biotin for 7 to 10 days at 25 °C.

For preparation and purification of nucleic acids, mycelium was collected on a Buchner funnel lined with a Miracloth filter, washed repeatedly with distilled water, and lyophilized. This was mixed with an extraction buffer containing 0·2 m-NaCl, 0·1 m-Tris–HCl, 0·002 m-EDTA, pH 7·0, at a ratio of 1·20 (w/v). Following the addition of bentonite (0·5 mg/ml), the
Table 1. Strains of *Endothia parasitica* used

<table>
<thead>
<tr>
<th>Original H strain*</th>
<th>Country of origin</th>
<th>H strain resulting from conversion of EP 155†</th>
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</thead>
<tbody>
<tr>
<td>EP 113</td>
<td>France</td>
<td>EP 713</td>
</tr>
<tr>
<td>EP 420</td>
<td>Italy</td>
<td>EP 747</td>
</tr>
<tr>
<td>EP 419</td>
<td>Italy</td>
<td>EP 779</td>
</tr>
<tr>
<td>EP 419</td>
<td>Italy</td>
<td>EP 780</td>
</tr>
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* Numbers refer to entries in the Culture Collection of the Connecticut Agriculture Experiment Station.
† Strain EP 155 of *E. parasitica* is a normal virulent strain of American origin.

The mixture was homogenized in a Braun model MSK cell homogenizer for 90 s using 30 g of glass beads. The homogenate was made 1% in SDS and emulsified with an equal volume of a mixture of distilled phenol (saturated with 0.1 M-Tris-HCl pH 8.0, and containing 0.1% hydroxyquinoline):chloroform:isoamyl alcohol (25:24:1, by vol.). After centrifugation at 10000 g for 30 min, the aqueous phase was re-extracted with an equal volume of chloroform:isoamyl alcohol (24:1).

Total nucleic acid (TNA) was precipitated by the addition of 2.5 vol. 95% ethanol to the final aqueous phase and kept at -20 °C overnight. The dsRNA was purified from TNA by selective precipitation in 4 M-LiCl (Diaz-Ruiz & Kaper, 1978) followed by chromatography on cellulose CF-11 (Morris & Dodds, 1979). Although the yields of dsRNA varied, H strains of European origin generally contained more dsRNA than those of American origin. The highest yield (180 μg dsRNA/g of lyophilized mycelium) was obtained from the French-derived H strain EP 713 and the lowest (16 μg dsRNA) was from the American H strain EP 868.

For preparation of hybridization probes, the dsRNA was further purified by centrifugation in linear-log density gradients (Brakke & Van Pelt, 1970), and partially hydrolysed using deionized formamide at 100 °C (Negruk *et al.*, 1980). The denatured dsRNA fragments were labelled to a specific activity of 10⁶ c.p.m./μg with [γ³²P]ATP using polynucleotide kinase (Maniatis *et al.*, 1982; Jordan & Dodds, 1983).

Dot blot hybridization was as described by Garger *et al.* (1983) using samples of 2 μl spotted directly onto dry nitrocellulose filters. The filters were autoradiographed for 16 to 48 h at -80 °C using Kodak XAR-5 X-ray film and Dupont Cronex Lightning Plus intensifying screens.

Electrophoretic analyses of dsRNA of French origin (EP 713), revealed four major bands (Fig. 1, lane 3). The mol. wt. of these dsRNA components were estimated previously (Dodds, 1980a) to be 4-6, 5-0, 5-9 and 6-2, all x 10⁶. The dsRNA of Italian origin showed either one major band and two intermediate ones (lanes 4 and 8) or two major bands (lane 5). The dsRNA from American H strains was resolved into a single major band and several minor bands (lanes 6 and 7). The mobilities of the slowest migrating bands in dsRNA of European origin were the same and were slightly less than that of dsRNA from American H strains.

The interrelationships among dsRNAs from European and American hypovirulent strains were determined by the dot blot hybridization technique. Denatured TNA or dsRNA from French, Italian and American sources as well as TNA from the dsRNA-free V strain was spotted on nitrocellulose filters and probed with ³²P-labelled fragments of denatured dsRNA from the French-derived H strain EP 713. The results (Fig. 2a) indicated that this probe hybridized with TNA or dsRNA from the same fungal strain (EP 713) as well as with dsRNA of Italian origin (H strains EP 747 and 780), but not with dsRNA from three American H strains (EP 915, 844 and 868) or with TNA from the V strain EP 155. This hybridization pattern was reproduced when comparable filters were probed with ³²P-labelled RNA fragments prepared from dsRNA of Italian origin, EP 779 (data not shown).

The ³²P-labelled RNA probe prepared from dsRNA of American origin (EP 915) hybridized strongly with dsRNA from three American H strains (EP 844, 868 and 915). However, little or no hybridization was detected with dsRNA from four European H strains or TNA from the V...
Short communication

Fig. 1. PAGE of dsRNA from several H strains of *E. parasitica* of European and American origins. Electrophoresis was in 5% polyacrylamide gels in 0.089 M-Tris, 0.089 M-boric acid, 0.002 M-EDTA, pH 8.0 at 50 V for 17 h (lanes 1 to 5) and 15 h (lanes 6 to 9). The gels were stained with ethidium bromide (4 μg/ml) and photographed using transmitted u.v. illumination and Polaroid Type 665 film. Lanes 1 and 9, dsRNA from *Helminthosporium maydis* virus, *Penicillium chrysogenum* virus and *P. stoloniferum* viruses S and F used as markers; lane 2, dsRNA from the 190S virus of *Helminthosporium victoriae* (mol. wt. 3.0 × 10^6); lane 3, dsRNA of French origin (EP 713); lanes 4 and 8, dsRNA of Italian origin (EP 747); lane 5, dsRNA of Italian origin (EP 779); lanes 6 and 7, dsRNA of American origin (EP 915 and EP 844, respectively). Numbers at the side of the gels are mol. wt. × 10^-6.

Fig. 2. Dot blot hybridization assay of denatured dsRNA and total nucleic acid (TNA) from several strains of *E. parasitica*. The nitrocellulose filters were probed with 32P-labelled fragments of denatured dsRNA of (a) French origin (EP 713) or (b) American origin (EP 915). The dotted samples were as follows: row 1, TNA from the dsRNA-free strain EP 155; rows 2 and 3, TNA and dsRNA, respectively, from the French-derived H strain EP 713; rows 4 and 5, dsRNA from Italian-derived H strains EP 747 and 780, respectively; rows 6, 7 and 8, dsRNA from American-derived H strains EP 915, 844 and 868, respectively. TNA was spotted in six dots in decreasing quantities (from left to right): 20, 10, 4, 2, 1 and 0.4 μg; dsRNAs were spotted in six dots in quantities of 500, 50, 10, 5, 2.5, and 1 ng.

These results provide the first direct evidence that dsRNAs from European H strains have sequences distinct from those of dsRNAs from American H strains. This is not surprising in
view of the known differences between these strains in phenotype and dsRNA electrophoretic patterns (Anagnostakis, 1982). The lack of cross-hybridization between dsRNA from European and American H strains suggests that they represent distinct virus-like agents rather than strains of the same agent. The dsRNAs from H strains from the same continent, on the other hand, were clearly closely related despite coming from widely separated locations, e.g. Michigan and Virginia. However, since the number of strains of either American or European origin examined so far is small, it is possible that H strains whose dsRNAs are unrelated may still be found in any one continent.

Even though the dsRNA of European origin showed three distinct electrophoretic banding patterns (Fig. 1), they exhibited significant sequence homology in the dot blot hybridization assay. This finding re-emphasizes that banding patterns should not be relied upon for studying interrelationships among dsRNAs (Van Alfen, 1982). The relatedness of dsRNA components present in an individual H strain has not yet been determined. It is not understood whether the multiple components of dsRNA within a single H strain represent a segmented genome of a single virus or a mixed infection with two or more viruses. Also, the possibility that only one dsRNA component is essential for replication and the others are satellites or deletion mutants cannot be ruled out. These interrelationships need to be investigated.

As information is gained on the molecular biology of dsRNA from various sources it may become desirable for the purpose of biological control to combine dsRNA of different origins in a single fungal strain. The lack of homology between dsRNA from European and American H strains suggests that these dsRNAs will not interfere with or exclude each other.

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