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# A cytoplasmically transmissible hypovirulence phenotype associated with mitochondrial DNA mutations in the chestnut blight fungus *Cryphonectria parasitica*

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ABSTRACT **Mutations causing mitochondrial defects were induced in a virulent strain of the chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr. Virulence on apples and chestnut trees was reduced in four of six extensively characterized mutants. Relative to the virulent progenitor, the attenuated mutants had reduced growth rates, abnormal colony morphologies, and few asexual spores, and they resembled virus-infected strains. The respiratory defects and attenuated virulence phenotypes (hypovirulence) were transmitted from two mutants to a virulent strain by hyphal contact. The infectious transmission of hypovirulence occurred independently of the transfer of nuclei, did not involve a virus, and dynamically reflects fungal diseases caused by mitochondrial mutations. In these mutants, mitochondrial mutations are further implicated in generation of the attenuated state by (*i*) uniparental (maternal) inheritance of the trait, (*ii*) presence of high levels of cyanide-insensitive mitochondrial alternative oxidase activity, (*iii*) cytochrome deficiencies, and (*iv*) structural abnormalities in the mtDNA. Hence, cytoplasmically transmissible hypovirulence phenotypes found in virus-free strains of *C. parasitica* from recovering trees may be caused by mutant forms of mtDNA.**

The ascomycetous fungus *Cryphonectria parasitica* is the causal agent of the blight responsible for the virtual disappearance of chestnut trees (*Castanea dentata*) from North American forests. Spontaneous natural control of this disease occurs in areas where the fungus is infected by hypovirulence-causing double-stranded RNA (dsRNA) viruses (1–3). However, some attenuated isolates from recovering chestnut trees exhibit cytoplasmically transmissible hypovirulence but do not contain dsRNAs (4, 5). Unlike virulent and dsRNA-containing hypovirulent strains, a high proportion of the virus-free hypovirulent isolates constitutively express high levels of cyanide (CN)-resistant, salicylhydroxamate-sensitive respiration (alternative oxidase activity) (5). Such strains resemble dsRNA-containing hypovirulent strains in that, relative to virulent strains, they grow more slowly, produce fewer conidia, and form morphologically abnormal colonies. These traits suggest that this type of hypovirulence may be affiliated with mitochondrial dysfunctions similar to those found in cytoplasmic mutants of well-characterized filamentous fungi (6, 7), particularly *Neurospora, Podospora*, and *Aspergillus* (8, 9). Mitochondria also might be involved in the attenuation of *Cryphonectria* and other fungi by prolonged asexual propagation in the laboratory (10). However, direct evidence for the association of mtDNA mutations with the spontaneous appearance of hypovirulence in dsRNA-free strains of *C. parasitica* is still lacking.

To explore the possible involvement of mitochondrial genes in the attenuation of virulence in *C. parasitica*, mutations that cause respiratory defects were induced and selected in a highly stable, virus-free virulent strain (Ep155). During the characterization of these mutants, we discovered that modified mtDNAs can cause a cytoplasmically transmissible hypovirulence phenotype similar to that found in strains infected by dsRNA viruses. The observations indicate that mtDNA mutations may cause hypovirulence in dsRNA-free populations of *C. parasitica* in healing cankers on chestnut trees and potentially can be used to control the morbidity of phytophathogenic fungi, particularly those that are resistant to or devoid of attenuating viruses.

## MATERIALS AND METHODS

**Fungal Strains, Culturing, and Crosses.** *C. parasitica* was grown and crossed as described (11, 12). Mutants were selected in a virulent strain of the A mating type, Ep155. Two strains of the a mating type, Ep339 and 80-2c, were used to test for sexual transmission of mutant phenotypes. Ep339 has nuclear DNAs and mtDNAs distinguishable from those of Ep155 by restriction fragment length polymorphism. Strain 80-2c has a nuclear mutation, *br*, which determines brown pigmentation of the mycelium and was derived by D. H. Huber in our laboratory from a strain provided by W. L. MacDonald (West Virginia University). A nuclear benomyl-resistant ($Bml^R$) mutant, EpBEN3, was selected from Ep155 for use as a recipient in assays for transmission of mutant phenotypes by hyphal contact. This mutant is as virulent as Ep155. Ep713, a virus-infected derivative of Ep155, served as a hypovirulent control in virulence tests.

**Mutagenesis and Selection of Mutants.** Mutations were induced by suspending conidia from the Ep155 strain in liquid medium (12) containing 1.0 mM ethidium bromide. After 5 h of incubation on a shaker, 5 ml of the suspension was dispensed into an open, sterile Petri dish and irradiated 20 min with UV light. The treatment killed 20–40% of the conidia. The treated conidia were diluted and spread on agar medium (12). Two days later, germlings were transferred to fresh medium (four per Petri dish). Colonies that grew more slowly than untreated Ep155 controls were screened for mitochondrial dysfunction.

**Respiration.** Cultures were started by inoculation of 3-mm squares of mycelium from colonies grown on agar medium into 125-ml flasks containing 30 ml of liquid medium. Mycelia were grown for 2 days in standing cultures and an additional day on a shaker set at 200 rpm. For measurement of oxygen consumption, each mat of mycelium was removed from the medium with a transfer needle, added to 5.0 ml of Vogel's (13) minimal medium containing 1% glucose, and homogenized for 5–10 s at medium speed with a Tekmar Tissuemincer. An appropriate aliquot of the homogenate was added to a chamber of a YSI 5300 biological oxygen monitor, adjusted to 3.0 ml with Vogel's glucose medium, and aerated 1 min with an aquarium pump. Alternative oxidase activity was measured as

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Abbreviations: $Bml^R$ and $Bml^S$, benomyl resistant and sensitive; ds, double stranded.
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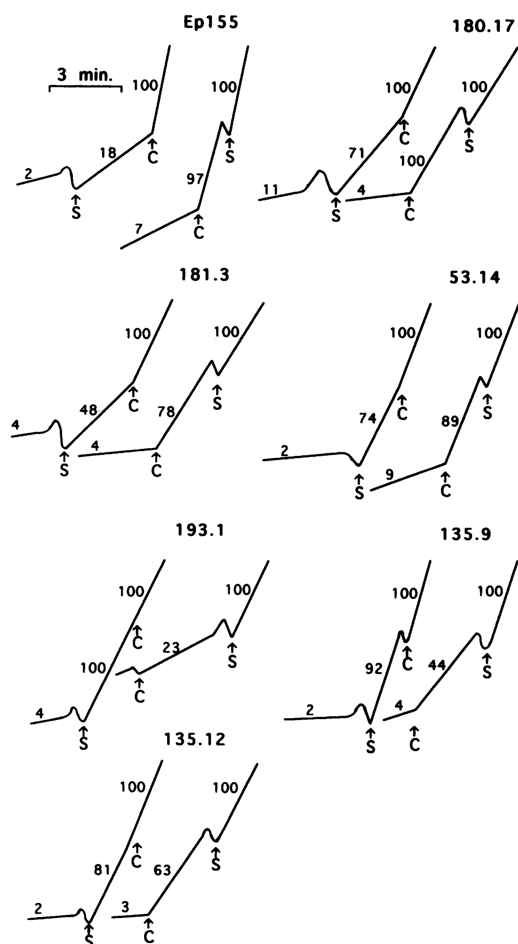


FIG. 1. Respiratory phenotypes of mutants derived from strain Ep155 of *C. parasitica*. Additions of CN and salicylhydroxamate are indicated by arrows C and S, respectively. Numbers above different segments of each tracing represent percentages of the initial respiratory activity (100%) remaining after addition of inhibitors. Initial respiratory activity represents the rate of oxygen consumption prior to the addition of any inhibitor. The rationale for this assay of respiratory phenotypes is provided in ref. 5.

described (5). KCN and salicylhydroxamate were added to the reaction mixtures to final concentrations of 1.0 and 4.16 mM, respectively.

**Virulence Tests.** Tests for virulence in apples (14) and on living trees (15) were performed as described.

**Transmission of Genetic Factors by Hyphal Contact.** Mycelial plugs from the donor and recipient (EpBEN3) strains were placed next to each other on agar medium near the wall of a Petri dish (2) and allowed to grow for 5 days. Plugs of mycelium were excised from the contact zone between the colonies and placed on medium containing Bml (0.5 $\mu$g/ml). Conidia from the resultant colonies were spread on medium without Bml and allowed to germinate 2 days before they were transferred individually to Petri dishes of agar medium. The resultant colonies were tested for resistance to Bml, for virulence, and for alternative oxidase activity. The criteria establishing cytoplasmic or nuclear location of genetic determinants in "heterokaryon tests" were described by Jinks (16) and Gillham (17).

**Cytochrome Spectra.** For determination of cytochrome content, mitochondria were prepared by differential centrifugation (18, 19) and processed as specified by Infanger and Bertrand (20) for recording difference spectra (19).

**Preparation and Analysis of mtDNA.** Mitochondria were purified by the flotation-gradient procedure (21), and mtDNA was prepared as described (22). In some cases, mtDNA was further purified by treatment with cetyltrimethylammonium bromide (23). Digestions of DNAs with restriction endonucleases and separation of DNA fragments by agarose gel electrophoresis were performed as recommended by Sambrook *et al.* (24).

## RESULTS

**Phenotypic Characteristics of the Mutants.** A screening of 4393 random isolates derived from mutagen-treated conidia yielded 250 colonies that grew more slowly than untreated isolates from the Ep155 strain. Six of the slow-growing isolates—135.9, 135.12, 193.1, 181.3, 180.17, and 53.14—were retained for further characterization because they had high levels of CN-resistant respiration (Fig. 1). Relative to the parental strain, mutants 135.9, 135.12, and 193.1 formed flat, highly pigmented mycelia with irregular edges (Fig. 2), whereas the colonies of 53.14 varied from small and severely diseased to normal in size and appearance. In contrast, the colonies of 180.17 and 181.3 were virtually indistinguishable from those of Ep155. A genotypic test based on nuclear DNA polymorphisms (25) confirmed that all six mutants are direct asexual descendants of Ep155 (data not shown). The results of virulence tests on apples and living trees indicated that there is a direct correlation between the aggressiveness of the mutants and their growth rates on complete medium (Fig. 2 and Table 1). In both tests, four of the six mutants (135.12, 135.9, 193.1, and 53.14) were hypovirulent relative to Ep155, whereas the two fast-growing mutants, 180.17 and 181.3, were as virulent as Ep155.

**Cytoplasmic Transmission of Respiratory Defects and Hypovirulence.** To determine the cytoplasmic or nuclear location of the genetic determinants for the CN-resistant respiratory phenotype and/or hypovirulence, each mutant was paired asexually with the Bml$^R$ EpBEN3 recipient to see if the phenotypes could be transmitted by hyphal contact. When the virulent EpBEN3 strain was paired with either 135.12 or 135.9, the majority of the recovered Bml$^R$ isolates had CN-resistant respiration (Table 2) and had become hypovirulent (the diameter of the necrotic spots formed on apples after 21 days of incubation varied from 1.47 to 4.28 cm compared to 6.55 cm for the virulent EpBEN3 control). Thus, the Bml$^R$ recipient had acquired the respiratory and virulence phenotypes from the Bml-sensitive (Bml$^S$) donors by hyphal contact, indicating that these properties are determined by cytoplasmic genetic factors, presumably mutant mtDNAs. When the 193.1, 180.17,

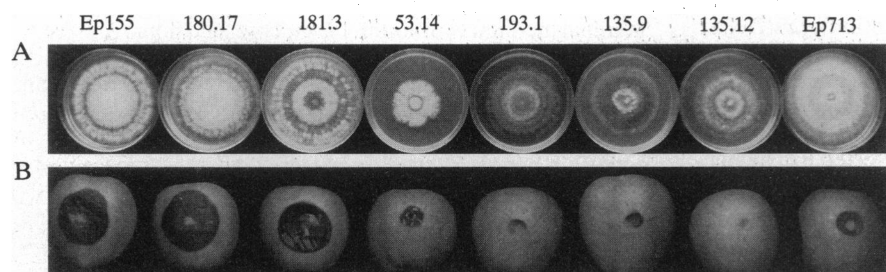


FIG. 2. Colony and virulence phenotypes of *C. parasitica* wild-type Ep155, cytoplasmic (135.9 and 135.12), and nuclear (53.14, 180.17, 181.3, and 193.1) respiratory mutants and Ep713, a virus-infected variant of Ep155 (3). (*A*) Colony phenotypes as manifested after 5 days of growth on complete agar medium under alternating 12-h periods of light and dark at room temperature. (*B*) Virulence on apples as manifested by sizes of necrotic spots at 14 days of incubation.

Table 1. Comparison of colony diameters and virulence on apples and living trees of wild-type Ep155, respiratory mutants, and Ep713, a virus-infected variant of strain Ep155

| Strains | Colony diameter, cm* | Apple test, cm* | Living tree, cm† |
|---|---|---|---|
| Ep155 | 6.77 ± 0.16 (a) | 6.55 ± 0.48 (a) | 3.06 ± 0.46 (a) |
| 181.3 | 6.87 ± 0.25 (a) | 5.48 ± 0.08 (a) | 3.18 ± 0.32 (a) |
| 180.17 | 5.97 ± 0.12 (b) | 6.13 ± 0.87 (a) | 2.89 ± 0.45 (a) |
| Ep713 | 5.63 ± 0.32 (bc) | 2.42 ± 0.75 (b) | — |
| 193.1 | 5.20 ± 0.10 (c) | 1.95 ± 0.80 (b) | 2.07 ± 0.35 (b) |
| 135.9 | 5.37 ± 0.15 (c) | 2.45 ± 0.43 (b) | 1.94 ± 0.28 (b) |
| 135.12 | 5.17 ± 0.15 (c) | 1.83 ± 0.43 (b) | 1.47 ± 0.20 (b) |
| 53.14 | 3.57 ± 0.21 (d) | 2.43 ± 0.08 (b) | 1.71 ± 0.32 (b) |

Diameters of colonies grown on agar medium were measured 5 days after inoculation. The diameter of necrotic spots formed after 21 days of incubation was used to measure virulence on apples. Virulence on living trees was assessed by measuring the diameters of lesions 30 days after inoculation. Dash indicates that, to avoid release of virus into a virus-free tree stand, the test was not done. Within each column, mean values followed by the same letters (a–d) were not significantly different from each other but were significantly different from means followed by a different letter according to Student's $t$ test.
*Mean ± SEM of three repeats.
†Mean ± SEM of six repeats.

and 181.3 mutants were paired with EpBEN3, none of the recovered Bml$^R$ and all of the Bml$^S$ single-conidial isolates had high levels of alternative oxidase (Table 2) and were hypovirulent. Therefore, the respiratory and hypovirulence characteristics of these mutants were not transferred from one nuclear background to another, as expected of a trait determined by a nuclear gene. Mutant 53.14 did not fuse with EpBEN3; hence, the location of the determinants of the respiratory and virulence phenotypes of this mutant could not be established through the heterokaryon test.

**Sexual Transmission of Respiratory and Virulence Traits.** Each mutant was crossed reciprocally with one or both of two stable virulent strains of the **a** mating type, Ep339 and 80-2c. At least forty spores from a minimum of four different perithecia from each cross were analyzed, and 1:1 segregation for brown and orange pigmentation was observed when 80-2c was one of the parents (Table 3). As expected of a cytoplasmically determined genetic trait, the CN-resistant respiratory phenotype was transmitted to almost all (103 of 108) the sexual progeny when mutant 135.9 was the female (protoperithecial) parent and to none of the progeny (0 of 130) when it was the male (conidial) parent (Table 3). In contrast, when the 135.12 mutant was the female parent, perithecia formed sparsely and the respiratory-deficiency phenotype did not reappear in the sexual progeny. When this mutant was crossed as a male with either Ep339 or 80-2c, none of the progeny had the slow-growth trait or CN-resistant respiration. In this respect, 135.12 behaves like the female-sterile "stopper" cytoplasmic mutants of *Neurospora crassa* (26). When such mutants are used as females in crosses, rare instances of late fertility do arise, but the mitochondrial phenotypes are not transmitted from the maternal parent to any of the progeny. The progeny of the cross between 135.12 and Ep339 were examined for mitochondrial restriction fragment length polymorphisms characteristic of the maternal and paternal parents (data not shown), and the results confirmed maternal transmission of the mitochondrial chromosome (27), even though the phenotype of the maternal parent did not reappear in any of the progeny. The CN-sensitive and CN-resistant respiratory phenotypes segregated 1:1 in the progeny from crosses of isolates 193.1, 180.17, 181.3, and 53.14 with either Ep339 or 80-2c, regardless of whether the mutant was the female or male parent (Table 3). Collectively, these observations confirmed the results of the heterokaryon tests, indicating that the respiratory defects and hypovirulence in 135.9 and 135.12 are caused by mutant alleles of cytoplasmic genes, whereas the same phenotypes of 193.1 and 53.14 and the respiratory defects of 180.17 and 181.3 result from nuclear gene mutations.

Table 2. Transmission of respiratory defects from Bml$^S$ donors to a Bml$^R$ recipient (EpBEN3) by hyphal contact

| Pair | Bml$^R$ | | Bml$^S$ | |
|---|---|---|---|---|
| | High AO | Low AO | High AO | Low AO |
| 135.12 + EpBEN3 | 10 | 0 | 0 | 0 |
| 135.9 + EpBEN3 | 26 | 0 | 1 | 0 |
| 193.1 + EpBEN3 | 0 | 7 | 7 | 0 |
| 180.17 + EpBEN3 | 0 | 10 | 2 | 0 |
| 181.3 + EpBEN3 | 0 | 3 | 7 | 0 |

Bml$^R$ and Bml$^S$, single-conidial isolates with high (>80% of total $O_2$ consumption) and low (<20% of total $O_2$ uptake) alternative oxidase (AO) activities, measured as CN-resistant respiration. Numbers represent the tally of single-conidial isolates recovered from each pairing that showed the phenotypes indicated. Values of zero indicate that no isolates of the particular phenotype were recovered, because selection of Bml$^R$ was applied to ensure recovery of the recipient.

Table 3. Maternal and Mendelian transmission of respiratory defects in sexual crosses

| | 80-2c | | | | Ep339 | |
|---|---|---|---|---|---|---|
| | High AO | | Low AO | | | |
| | Orange | Brown | Orange | Brown | High AO | Low AO |
| ♀ parent | Second parent as ♂ | | | | | |
| 135.9 | 51 | 52 | 2 | 3 | — | — |
| 135.12 | — | — | — | — | 0 | 77 |
| 53.14* | — | — | — | — | 13 | 27 |
| 193.1 | 12 | 12 | 11 | 13 | 25 | 25 |
| 181.3 | 17 | 17 | 8 | 18 | 23 | 27 |
| 180.17 | 10 | 14 | 15 | 10 | 22 | 28 |
| Ep155 | 0 | 0 | 27 | 23 | 0 | 47 |
| ♂ parent | Second parent as ♀ | | | | | |
| 135.9 | 0 | 0 | 41 | 29 | 0 | 50 |
| 135.12 | 0 | 0 | 23 | 26 | 0 | 59 |
| 53.14* | — | — | — | — | 27 | 23 |
| 193.1 | 9 | 5 | 13 | 23 | — | — |
| 181.3 | 10 | 11 | 16 | 10 | — | — |
| 180.17 | 5 | 7 | 12 | 14 | 18 | 22 |
| Ep155 | 0 | 0 | 24 | 25 | 0 | 31 |

Each respiratory mutant was crossed reciprocally with 80-2c and/or Ep339 (second parent). The level of alternative oxidase (AO) activity was used as the indicator of respiratory phenotype as described in Table 2. Dashes indicate that crosses were sterile or were not done.
*Sexually incompatible with 80-2c for unknown reason.

**Mitochondrial Cytochrome Complements.** In *Neurospora*, the induction of the alternative oxidase by mtDNA or nuclear gene mutations commonly is associated with a deficiency in one or more of the mitochondrial cytochromes (28, 29). The composition of the mitochondrial cytochrome complement in mitochondria from Ep155 and the mutants derived from this strain could not be determined spectrophotometrically because of interference from pigments. However, this interference was minimized in mitochondria from strains that had inherited the *br* allele from 80-2c in crosses. Unfortunately, infertility or incompatibility prevented the construction of *br* derivatives of mutants 135.12 and 53.14 (see Table 3). Relative to the *br* "wild type," 135.9 is deficient in cytochrome *a* (Fig. 3), whereas 180.17, 181.3, and 193.1 are severely or partially deficient in cytochromes *a* and *b* (data not shown). On the basis of their genetic and phenotypic properties, we propose to name the 135.9 and 135.12 mitochondrial mutants *mit1* and *mit2*, respectively.

**Rearrangements in the mtDNAs of Cytoplasmic Mutants.** Since the cytoplasmically inherited senescence syndromes of

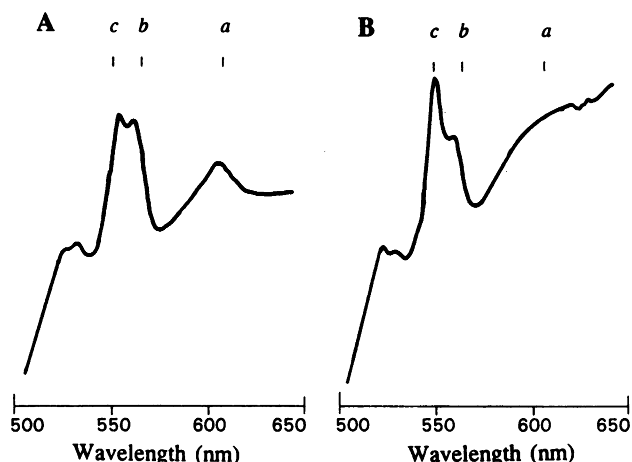


FIG. 3. Cytochrome spectra obtained by recording the difference in absorbance between dithionite-reduced and ferricyanide-oxidized mitochondria at wavelengths of light ranging from 500 to 650 nm. (*A*) 80-2c. (*B*) 135.9. Absorbance maxima at 550, 560, and 608 nm correspond to $\alpha$ peaks of cytochromes *c*, *b*, and *a*, respectively.

*Neurospora*, *Podospora*, and *Aspergillus* are caused by the gradual accumulation-defective derivatives of mitochondrial chromosomes (7), the number and sizes of the *Hpa* II and *Bgl* II restriction fragments of the mtDNAs from the respiratory mutants were compared with those of the mtDNA from Ep155 (Fig. 4). An unusual, small plasmid-like DNA frequently appears in high amounts in the mtDNAs of *mit1* and *mit2* cytoplasmic mutants, but it has never been observed in mtDNA preparations from Ep155. Nevertheless, this DNA hybridizes with a segment of the mtDNA of Ep155. Therefore, the plasmid-like element seems to be a derivative of the mitochondrial chromosome similar to the *sen* DNAs that frequently emerge in senescing cultures of *Podospora anserina* (30). In contrast, the mtDNAs from the nuclear respiratory mutants did not differ noticeably from the mtDNA of Ep155 (data not shown).

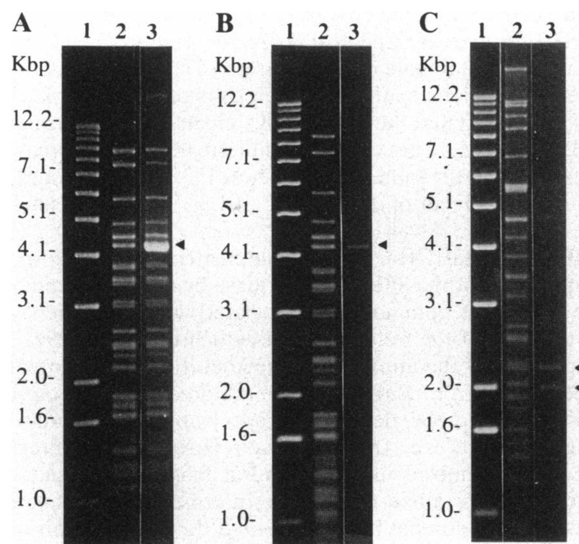


FIG. 4. Abnormal mtDNAs in hypovirulent, cytoplasmic respiratory mutants of *C. parasitica*. Amplified unusual restriction fragments are marked by arrowheads on the right. Lanes 1, dsDNA size markers of lengths indicated in kbp on the left. (*A*) Comparison of *Hpa* II restriction patterns of the mtDNAs from Ep155 (lane 2) and *mit2* (135.12, lane 3). (*B*) Comparison of *Hpa* II restriction patterns of the mtDNAs from Ep155 (lane 2) and *mit1* (135.9, lane 3). (*C*) Comparison of *Bgl* II restriction patterns of the mtDNAs from Ep155 (lane 2) and *mit1* (135.9, lane 3).

## DISCUSSION

The association of attenuated virulence phenotypes in *C. parasitica* with the presence of RNA viruses, particularly those of the hypovirus type, is the paradigm for the causation of hypovirulence by cytoplasmically transmissible genetic factors in phytopathogenic fungi (3, 31, 32). Therefore, the appearance of cytoplasmically transmissible forms of hypovirulence in *C. parasitica* strains devoid of viruses was a paradox, particularly since they were isolated from healing cankers on chestnut trees (4, 5). The frequent appearance of elevated alternative oxidase activity in the virus-free, attenuated isolates implicated mitochondria in the generation of hypovirulence phenotypes (5). In isolates derived from trees, a direct connection between the attenuated virulence phenotypes and mtDNA mutations cannot be established easily because *C. parasitica* has a rather large and highly variable mtDNA (27). Since a precise genetic or physical map has not been generated yet, and the virulent progenitors of hypovirulent isolates cannot be identified in a natural setting, it is virtually impossible to separate innocuous polymorphisms from deleterious deletions or insertions. Therefore, we have resorted to the induction of mutations in a laboratory strain to demonstrate that the infectious hypovirulence that appears in virus-free isolates from healing cankers can be caused by mtDNA mutations. We refer to this type of hypovirulence as "mitochondrial hypovirulence" to distinguish it from hypovirulence elicited by viral infections.

The asexual transmission pattern of mitochondrial hypovirulence is similar to the pattern of transmission characteristic of hypovirulence phenotypes caused by extramitochondrial mycoviruses. Although the mitochondria of the *mit1* and *mit2* mutants potentially are as infectious as the hypoviruses (2, 3) in contacts between vegetatively compatible strains, nothing is known about their transmission by contact between strains that belong to different compatibility groups. However, there is an important difference in the transmission of mitochondria and viruses: the extramitochondrial viruses rarely are transmitted sexually (3), whereas mitochondrial hypovirulence can be transmitted from the maternal parent to all the progeny. Therefore, the dispersal of this type of hypovirulence should be less dependent on vegetative compatibility than the dispersal of most mycoviruses, enhancing its potential as an alternative and supplementary approach to virus-based programs (3, 33) for controlling fungal diseases, particularly those caused by fungi devoid of attenuating viruses.

The attenuation of virulent strains by hyphal contact with either 135.9 or 135.12 indicates that the *mit1* and *mit2* mtDNA mutations are "suppressive." Suppressiveness is a property associated with mtDNA mutations in a variety of fungi and is manifested as a gradual amplification of mutant mitochondrial chromosomes and the concomitant decline in amount of wild-type mtDNA in vegetatively propagated heteroplasmons. In the filamentous fungi, suppressiveness is characteristic of mtDNA mutations that disrupt cytochrome-dependent electron-transport activity, regardless of the nature of the genetic change (9). In *Neurospora* alone, the process is elicited by point mutations, large deletions, and disruption of mitochondrial genes by the integration of plasmids into the mtDNA (7–9). Thus, it appears that the hypovirulence associated with the *mit1* and *mit2* mutations is infectious because the functionally crippled mitochondria that are transferred from the donor through naturally occurring hyphal anastomoses proliferate rapidly in the hyphae of the virulent recipient and cause attenuation as they replace the normal organelles.

The generation of a cytoplasmically transmissible form of hypovirulence by mutation of the mitochondrial chromosome

also may occur in *Ophiostoma ulmi*, the causative agent of Dutch elm disease. Cytochrome oxidase deficiencies (34) and abnormal mtDNAs (35, 36) have been observed in a dsRNA-containing hypovirulent variant of this fungus. However, it remains unclear if the aggressiveness of this fungus is diminished by the presence of the dsRNA or by the appearance of abnormal forms of mtDNA. Furthermore, the etiology of the hypovirulence phenotype in *Ophiostoma* is confounded by the fact that the virulent and hypovirulent variants are considered to be different, although closely related, species of the same genus—namely, *O. ulmi* (hypovirulent) and *Ophiostoma novo-ulmi* (virulent) (36). A dsRNA similar to that found in the mitochondria of *O. ulmi* was discovered recently in the mitochondria of a moderately hypovirulent strain of *C. parasitica* (37), but there is no indication that this element in any way affects the respiratory proficiency of the organelles.

The correlation of the degree of virulence with the growth rate of the cytoplasmic and nuclear respiratory mutants supports the notion that the attenuated phenotype is caused by metabolic debilitation rather than silencing of specific virulence genes, at least in *C. parasitica* (3, 5). In this respect, it is worth noting that reduced virulence frequently is associated with nuclear mutations affecting the growth and/or morphology of the fungus (38). The debilitation of *C. parasitica* by hypoviral infection also correlates with changes in the expression of several nuclear genes (3) and most notably involves the silencing of genes coding for laccase (39, 40) and cutinase (41). Whether or not similar pleiotropic effects are involved in the expression of mitochondrial hypovirulence remains to be established.

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1. Anagnostakis, S. L. (1987) *Phytopathology* **79,** 23–37.
2. MacDonald, W. L. & Fulbright, D. W. (1991) *Plant Dis.* **75,** 656–661.
3. Nuss, D. L. (1992) *Microbiol. Rev.* **56,** 561–576.
4. Fulbright, D. W. (1990) in *New Directions in Biological Control*, eds. Baker, R. & Dunn, P. (Liss, New York), pp. 693–702.
5. Mahanti, N., Bertrand, H., Monteiro-Vitorello, C. B. & Fulbright, D. W. (1993) *Physiol. Mol. Plant Pathol.* **42,** 455–463.
6. Bertrand, H., Collins, R. A. L., Stohl, L., Goewert, R. & Lambowitz, A. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6032–6036.
7. Griffiths, A. J. F. (1992) *Annu. Rev. Genet.* **26,** 351–372.
8. Bertrand, H. & Griffiths, A. J. F. (1989) *Genome* **31,** 155–159.
9. Bertrand, H. (1995) *Can. J. Bot.*, in press.
10. Naiki, T. & Cook, R. J. (1993) *Phytopathology* **73,** 1652–1656.
11. Anagnostakis, S. L. (1979) *Mycologia* **71,** 213–215.
12. Puhalla, J. E. & Anagnostakis, S. L. (1971) *Phytopathology* **61,** 169–173.
13. Vogel, H. J. (1956) *Microb. Genet. Bull.* **13,** 42–43.
14. Fulbright, D. W. (1984) *Phytopathology* **74,** 722–724.
15. Elliston, J. E. (1978) in *Proceedings of The American Chestnut Symposium*, eds. MacDonald, W. L., Cech, F. C. & Smith, H. C. (West Virginia Univ. Books, Morgantown), pp. 95–100.
16. Jinks, J. L. (1964) *Extrachromosomal Inheritance* (Prentice Hall, Englewood Cliffs, NJ).
17. Gillham, N. W. (1978) *Organelle Heredity* (Raven, New York).
18. Bertrand, H. & Pittenger, (1969) *Genetics* **61,** 643–659.
19. Bertrand, H. & Pittenger, T. H. (1972) *Genetics* **71,** 521–533.
20. Infanger, A. & Bertrand, H. (1986) *Curr. Genet.* **10,** 607–617.
21. Lambowitz, A. M. (1979) *Methods Enzymol.* **59,** 421–432.
22. Vierula, P. J. & Bertrand, H. (1992) *Mol. Gen. Genet.* **234,** 361–368.
23. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1987) *Current Protocols in Molecular Biology* (Greene and Wiley, New York).
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 26.
25. Milgroom, M. G., Lipari, S. E. & Powell, W. A. (1992) *Genetics* **131,** 297–306.
26. Bertrand, H., Szakacs, N. A., Nargang, F. E., Zagozeski, C. A., Collins, R. A. & Harrigan, J. C. (1976) *Can. J. Genet. Cytol.* **18,** 397–409.
27. Milgroom, M. G. & Lipari, S. E. (1993) *Phytopathology* **83,** 563–567.
28. Lambowitz, A. M. & Zannoni, D. (1978) in *Plant Mitochondria*, eds. Ducet, G. & Lance, C. (Elsevier/North-Holland, Amsterdam), pp. 283–291.
29. Bertrand, H., Argan, C. A. & Szakacs, N. A. (1983) in *Mitochondria 1983*, eds. Schweyen, R. J., Wolf, K. & Kaudewitz, F. (deGruyter, Berlin), pp. 495–507.
30. Dujon, B. & Belcour, L. (1989) in *Mobile DNA*, eds. Berg, D. E. & Howe, M. M. (Am. Soc. for Microbiol., Washington, DC), pp. 861–878.
31. Grente, J. & Sauret, S. (1969) *C.R. Hebd. Seances Acad. Sci.* **268,** 2347–2350.
32. Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L. & Day, P. R. (1975) *Science* **189,** 890–891.
33. Chen, B., Choi, G. H. & Nuss, D. L. (1994) *Science* **264,** 1762–1764.
34. Rogers, H. J., Buck, K. W. & Brasier, C. M. (1987) *Nature (London)* **329,** 558–560.
35. Charter, N. W., Buck, K. W. & Brasier, C. M. (1993) *Curr. Genet.* **24,** 505–514.
36. Brasier, C. M., Bates, M. R. & Charter, N. W. (1993) in *Dutch Elm Disease Research*, eds. Sticklen, M. B. & Sherald, J. L. (Springer, New York), pp. 308–321.
37. Polashock, J. J. & Hillman, B. I. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8680–8684.
38. Anagnostakis, S. L. (1984) *Phytopathology* **74,** 561–565.
39. Rigling, D. & Van Alfen, N. K. (1991) *J. Bacteriol.* **173,** 8000–8003.
40. Choi, G. H., Larson, T. G. & Nuss, D. L. (1992) *Mol. Plant Microbe Interact.* **5,** 119–128.
41. Varley, D. A., Podila, G. K. & Hiremath, S. T. (1992) *Mol. Cell. Biol.* **12,** 4539–4544.