Characterization of Double-stranded RNA-free Hypovirulent Strains of *Cryphonectria parasitica*

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ABSTRACT. Hypovirulent strains of Cryphonectria parasitica without detectable levels of double-stranded RNA have been associated with nonlethal cankers in Michigan American chestnut stands. This indicates that transmissible hypovirulence can be induced by cytoplasmic elements other than dsRNA. A consistent feature of dsRNA-free hypovirulent strains of *C.* parasitica is that a large portion of the respiratory activity of the mycelium is cyanide-resistant; whereas the respiration of virulent strains is cyanide-sensitive. By analogy with other fungal systems such as *Neurospora,* the induction of a cyanide-resistant alternative oxidase activity is symptomatic of mitochondrial mutations that affect the activity of the cytochrome-dependent mitochondrial electron transport system. These naturally occurring dsRNA-free isolates are described and compared to laboratory derived isolates with reduced virulence.

The biological control of chestnut blight, known as hypovirulence, is common and widespread in Michigan (10). Genetic and correlative evidence strongly suggests that double-stranded RNA (dsRNA) is a cytoplasmic transmissible element that, at least indirectly and possibly directly, is responsible for hypovirulence in Michigan populations of *Cryphonectria parasitica* (Murr.) Barr (8, 13, 15). In Michigan, hypovirulent strains are quite variable. The variability probably arises from the different dsRNA homology groups (10, 16), the number of dsRNA genomes infecting a strain (7) and the cytoplasmic and nuclear diversity of the fungal isolate. To add to the diversity of hypovirulent phenotypes found in Michigan, recent experiments in our laboratories indicate that hypovirulence also can be induced by genetic elements other than dsRNA (9, 14).

The attenuation of virulence in some strains by cytoplasmic factors other than dsRNA implies that another determinant of hypovirulence could be dysfunctional mitochondria similar to those found in respiration-related degenerative processes associated with stopper, senescence and ragged variants of *Neurospora, Podospora* and *Asper-* , respectively (4, 5). To date, studies on only one fungal phytopathogen relate respiration to a reduced-virulence phenotype and morphological debilitation (18). Hypovirulent strains of *Ophiostoma ulmi* (Buism.) Nannf. contain dsRNA "infected" mitochondria that produce extremely low levels of cytochrome aa3, the terminal electron acceptor. It was speculated by the authors that an alternate oxidase may function as the terminal electron acceptor.

The mitochondria of many fungi and higher plants have a branched-chain electron transport system in which one branch is a conventional cytochrome system and the other is an alternative, cyanide (CN)-resistant, salicyl hydroxamate (SHAM)-sensitive pathway (12). Electron transport in these systems branch and the resulting alternative pathway is not linked to oxidative phosphorylation. The function of the CN-resistant, SHAM-sensitive pathway of mitochondrial respiration in fungi is unknown, but it is induced when respiration through the cytochrome system is impaired. In *Neurospora,* high levels of cyanide-resistant respiration in a fungal culture can be used as a diagnostic indicator of respiratory problems (6, 11, 12).

The establishment of a direct genetic or physiological link between respiratory defects and the attenuation of virulence in dsRNA-free strains of *C. parasitica* would considerably widen our understanding of how the hypovirulence phenotype may function as a biocontrol process in Michigan and possibly elsewhere. Therefore, we describe in this paper *C. parasitica* strains that appear hypovirulent yet lack detectable levels of dsRNA.

MATERIALS AND METHODS

Strains. The strains or isolates of C. *parasitica* used in this study are listed and described in Table 1. Cultures were maintained under fluorescent light on potato dextrose agar (PDA; Difco, Detroit, Mich.) at 20 C. Broth cultures were grown at room temperature for 5 days in the *Endothia* complete liquid medium (ECM) of Puhalla and Anagnostakis (17) modified by the omission of glucose.

Respiration. The respiration of whole mycelium was measured polarographically using a Clark electrode and a YSI Model 53 biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio). Respiration was measured using modifications of the basic techniques previously described for *Neurospora* by Lambowitz and Slayman (11). Five-day-old cultures grown in ECM were homogenized for 15-20 sec using a Polytron homogenizer (Biospec Products, Racine, Wis.). The reaction was initiated by pipetting 3 ml of the homogenized mycelium into a reaction chamber. The sample was aerated after which the electrode was quickly inserted into the reaction chamber and the oxygen consumption recorded. The reaction

Table 1. Strains used in this study.

Strains	Description
Ep155	Standard virulent strain from Connecticut
CL1-16	Virulent strain from Michigan
CL25	dsRNA-free hypovirulent strain from northern Michigan
CL25-4	Virulent single-conidial-isolate (sci) of CL25
CL25-9	dsRNA-free hypovirulent sci of CL25
14-B	dsRNA-free hypovirulent strain from southern Michigan
4-C	Virulent strain from Michigan
CL1-16.3	Sector of CL1-16 with dark-pigmented morphology and less virulent

chamber was maintained at 25 C and the mycelial suspension was constantly mixed with a magnetic stirring bar.

Potassium cyanide and salicyl hydroxamic acid (SHAM), respiratory inhibitors of the cytochrome chain and the alternate oxidase pathways, respectively, were used to analyze the capacities of the cultures to respire through each pathway. The inhibitors were injected separately into the reaction chamber during measurements of oxygen consumption.

Sexual crosses and single-spore isolation. Sexual crosses were performed on water agar and chestnut wood in sterile culture dishes as previously described (3). Single ascospores and conidia were subcultured after serial dilution on water agar and PDA, respectively.

Isolation of sectors. CL1-16, when grown on PDA in the laboratory, frequently produces sectors as it grows toward the edge of the culture dish. These sectors were subcultured from CL1-16 and characterized for virulence, stability of morphology and vegetative transfer of the phenotype.

Virulence and transmission assays. Virulence of isolates was determined in apple fruit (8) and dormant chestnut wood virulence assays. The transfer of morphological characteristics and/or hypovirulence was accomplished by pairing isolates side-by-side in a culture dish as described by Anagnostakis (1).

RESULTS AND DISCUSSION

To determine if dsRNA-free C. parasitica isolates expressing the hypovirulence phenotype were affected by mitochondria) impairments, respiration was measured and compared among hypovirulent and virulent strains. The cyanide-resistant, alternate oxidase pathway represented a higher percentage of the total respiration in the dsRNA-free hypovirulent strains than other strains (Table 2). These findings indicate that dsRNA-free hypovirulence may be the result of respiratory dysfunction that induces high levels of alternate oxidase activity. When CL25-9 was paired with virulent strain 4-C, 4-C was converted to the hypovirulent phenotype and alternate oxidase activity increased demonstrating the concerted transfer of hypovirulence and alternate oxidase activity (Table 2). The hypovirulence phenotype in CL25 also was transmitted to a genetically marked strain of CL1-16.

Table 2. Alternate oxidase pathway as a percentage of total respiration in different strains of *Cryphonectria parasitica*.

Strain	Phenotype	Alternate oxidase as % of total respiration
Ep155	Virulent	9.0
CL1-16	Virulent	10.5
CL25-9	Hypovirulent	72.0
CL25-4	Virulent	14.0
4-C	Virulent	8.0
4-C(H)	4-C converted to hypovirulent after pairing with CL25-9 hypovirulent	82.0
14-B	Hypovirulent	85.0

Upon single conidial isolation, CL25 produced 80% virulent isolates (CL25-4) and 20% hypovirulent isolates (CL25-9). Continued isolation of conidia from CL25-4 yielded only virulent isolates, but segregation of hypovirulent to virulent and hypovirulent continued upon single-conidial isolation of CL25-9. The 4:1 segregation ratio of virulence to hypovirulence observed in CL25 and CL25-9 also was found to occur in the genetically marked CL1-16 strain converted to hypovirulent by CL25-9.

Segregation ratios of hypovirulent and virulent upon single-conidial isolation of 14-B, a dsRNA-free hypoviru lent strain from Kellogg Forest, Mich., was not as clear-cut. Single-conidial isolates varied in morphology and virulence upon continuous subculture. Some conidia germinated and grew into micro-colonies during single-conidial isolation procedures.

Hypovirulent isolates with abnormal culture morphology and reduced virulence were selected from the virulent strain CL1-16 by subculturing mycelium isolated from the sector. One sector in particular, CL1-16.3, has been stable upon subculture showing both a dark pigmentation and reduced virulence. As far as we know, this isolate never contained dsRNA, a prerequisite for flat mutations (2). This isolate, when paired with CL1-16, alters the color, morphology and virulence of CL1-16. The portion of CL1-16 that appears to be converted by CL1-16.3 remained altered in morphology and reduced in virulence upon subculturing indicating the genetic transfer of an unknown "virulence inhibition factor" (10) during hyphal fusion.

Unlike CL25, where hypovirulence is only maternally inherited during sexual crosses, hypovirulence, dark pigmentation and the altered morphology of CL1-16.3 has, so far, only infrequently been paternally inherited demonstrating non-Mendelian ratios. It is possible that the genetic factor responsible for the unique phenotype of the sector induces a female sterile genotype as is observed in other fungi with known genetic impairments.

If the preliminary results with the sector of CL1-16 are reproducible, studies on the origin and development of the sector phenotype may help us understand how isolates lose virulence after continuous subculturing in the laboratory.

It also may help us determine the origin of dsRNA-free hypovirulence in Michigan and the significance of dsRNA-free hypovirulence for the biocontrol of chestnut blight.

The relationship among CL25, 14-B and CL1-16.3 is not entirely clear except that they are all phenotypically hypovirulent without having detectable levels of dsRNA. The relationship between CL1-16.3 and flat mutants described by Anagnostakis (2) also is of interest. By comparing different forms of hypovirulence, including dsRNA-associated hypovirulence with dsRNA-free hypovirulence, we can begin to learn about the complex genetic matrix required for the expression of full virulence and the diverse genetic avenues available for restricting that expression.

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