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The Role of Mitochondria in the Hypovirulence of Chestnut Blight Fungus Cryphonectria Parasitica

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THE ROLE OF MITOCHONDRIA IN THE HYPOVIRULENCE OF CHESTNUT BLIGHT FUNGUS CRYPHONECTRIA PARASITICA.

By

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ABSTRACT

THE ROLE OF MITOCHONDRIA IN THE HYPOVIRULENCE OF CHESTNUT BLIGHT FUNGUS CRYPHONECTRIA PARASITICA.

by

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Hypovirulent strains of Cryphonectria parasitica have been associated with the presence of double-stranded ribonucleic acid (dsRNA). Hypovirulence can be cytoplasmically transferred to virulent strains through hyphal anastomosis. Strain CL25 from Crystal Lake, Michigan, has many characteristics of dsRNA-associated hypovirulence but harbors no detectable amounts of dsRNA. Genetic studies of CL25 showed that the hypovirulent phenotype is carried in 20% of the conidia, maternally inherited in sexual crosses, and can be transferred to other virulent strains via hyphal fusion. This suggests that there is a cytoplasmic "virulence inhibition factor" (VIF) other than dsRNA responsible for hypovirulence in CL25.

Since hypovirulence in CL25 is maternally inherited, several studies were initiated to investigate the possibility that mitochondria might be involved. Two low copy number plasmids of 6 and 10 kilobase pairs (kb) in size were discovered in the mitochondria of CL25. A portion of the 6 kb plasmid was cloned and was used to screen other strains. The plasmid was detected in all virulent and hypovirulent strains of C. parasitica screened, and thus, the 6 kb plasmid could not be directly involved in the hypovirulence of CL25. Thus far, the function of this plasmid remains unknown.

Respiration studies of CL25 and other dsRNA-free hypovirulent strains revealed that the cyanide-insensitive or alternate oxidase pathway accounted for as much as 85% of the total

respiration in these strains, compared to 8% in virulent and 16% in dsRNA-associated hypovirulent strains. This study suggests that a respiratory defect may be closely associated with dsRNA-free hypovirulence.

Using RFLPs and chloramphenicol resistance as markers for the mitochondrial genome and pigment production as a marker in the nuclear genome, it was possible to demonstrate that mitochondria can transfer from a dsRNA-free hypovirulent strain to a virulent strain. The converted strain obtained the hypovirulence and chloramphenicol-resistance traits of CL25, but retained the pigmentation phenotype of the virulent strain, and contained the mitochondrial RFLPs of both strains. The alternate oxidase pathway accounted for 82% of total respiration in the converted strain as compared to 8% before conversion indicating concerted cytoplasmic transfer of mitochondria, the hypovirulent phenotype and alternate oxidase activity. This supports the hypothesis that mitochondria are associated with the dsRNA-free hypovirulence phenotype.

Dedicated in the memory of my parents,

Mr. Nishamani Khuntia

and

Mrs. Shantisudha Khuntia

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LITERATURE REVIEW

Cryphonectria (= Endothia) parasitica (Murr) Barr. is the fungal phytopathogen responsible for chestnut blight, the disease which has virtually eliminated the American chestnut (Castanea dentata (Marsh) Borkh) from the eastern deciduous forests of North America. Hypovirulent strains of this fungus have resulted in a natural biocontrol of the disease (MacDonald and Fulbright, 1991). Hypovirulence of C. parasitica is described as a cytoplasmically transmissible trait that results in attenuation of virulence of the pathogen (Van Alfen et al, 1975). Double-stranded RNA (dsRNA) genetic elements have been associated with the phenomenon of "transmissible hypovirulence".

The History of Chestnut blight

Chestnut blight remains among the most devastating plant diseases known. It has eliminated the American chestnut as a major tree species in eastern North America. The first infected trees were reported in 1904 in the Bronx zoo (Anagnostakis, 1982). The fungus was apparently introduced into New York city and Long Island on nursery stock of oriental chestnuts, possibly from Japan. Chinese chestnut (C. mollissima) and Japanese chestnut (C. crenata, Sieb. and Zucc.) exhibit a high level of tolerance to the blight, but these chestnut species can act as carriers of the fungus. The American chestnuts proved to be very susceptible and the fungus spread quickly throughout the natural range of the chestnut.

The most obvious symptoms of chestnut blight induced by virulent strains of C. parasitica are yellowing and wilting of the foliage of a branch or main stem. The fungus infects through wounds and induces cankers that girdle the trunk or branch, affecting the vascular system. The root system remains alive, but sprouts growing from the base of the stump are eventually killed by subsequent infection.

In 1938, Biraghi found the fungus on European chestnut trees (C. sativa Mill) in northern Italy and within a decade the disease spread and killed trees in many areas. In 1951, Biraghi noted that some coppice trees were recovering from blight in an area where blight was observed in 1938.

The phenotype of these newly discovered strains was termed "hypovirulent" by a French mycologist J. Grente in the early 1960's. Hypovirulent strains of the fungus were described as white in culture and reduced in virulence. It was found that these characteristics could be cytoplasmically transmitted to virulent strains of C. parasitica via hyphal fusion. Day et al (1977) discovered the presence of double-stranded RNA (dsRNA) in hypovirulent strains and differentiated hypovirulent strains from virulent strains by the lack of dsRNA in virulent strains. When the hypovirulence trait was transmitted to a virulent isolate, the dsRNA was also transmitted (Anagnostakis and Day, 1979; Day et al. 1977). The discovery of hypovirulence gave hope that biological control of blight with hypovirulence might result in the recovery of both the American chestnut in eastern North America.

Biology and Genetic organization of dsRNA

The cytoplasmic nature of hypovirulence was first proposed by Grente and Sauret. Van Alfen et al (1975) first genetically demonstrated the cytoplasmic mode of hypovirulence transfer by pairing genetically marked, auxotrophic strains of C. parasitica in chestnut bark

and in culture. A hypovirulent lysine auxotroph was paired with a virulent methionine auxotroph in the trunks of American chestnut seedlings. Ninety days later, methionine auxotrophs isolated from the canker were hypovirulent. Conversion of the methionine requiring strain from virulent to hypovirulent indicated that the hypovirulent phenotype was transferred from the lysine auxotrophs. More evidence for the cytoplasmic nature of hypovirulence was obtained when heterokaryons were formed after pairing hypovirulent methionine and virulent arginine auxotrophs. From single-conidial isolates obtained from the heterokaryon, hypovirulent strains were obtained which required both methionine and arginine.

The ability of hypovirulent isolates to transfer the factors responsible for hypovirulence to virulent isolates has been described as transmissible hypovirulence. The transfer of hypovirulence to virulent isolates occurs between compatible isolates of the fungus through hyphal fusion (Newhouse et al. 1983). In a compatible reaction, the hyphae of two isolates fuse and cytoplasmic exchange occurs. Hyphal fusion is controlled by vegetative compatibility (VC) genes. Anagnostakis and Day (1979) have estimated that there are at least five VC genes, and all alleles must be the same at these loci for stable fusion to occur. Cytoplasmic transfer of dsRNA is possible if all the alleles are not the same, however, cytoplasmic transfer of dsRNA has been shown to occur more frequently when the alleles are homologous and is reduced in frequency if the alleles are heterologous. Therefore, it appears that the more stable the hyphal fusion, the more likely the occurrence of cytoplasmic transfer.

Correlative evidence indicating the involvement of dsRNA in hypovirulence was strengthened by a dsRNA curing experiment conducted by Fulbright (1984). Through the application of cycloheximide, hypovirulent C. parasitica isolates were cured of their dsRNA and the cured isolates had increased virulence. These cured strains could be converted back to the hypovirulent form by fusion with a hypovirulent strain.

Double-stranded RNAs associated with different hypovirulent strains vary considerably with respect to size, number of species, concentration and sequence homology. (Dodds, 1980; Elliston, 1985; Garrod et al, 1985; Paul and Fulbright, 1988). There is no consensus yet as to what constitutes the minimum dsRNA genome of any of the hypovirulent strains. A study by Paul and Fulbright (1988), using dsRNA from different geographical locations showed that there was no homology between Michigan dsRNA and dsRNA from West Virginia and other North American locations. Hypovirulent strains from Michigan, however, in spite of the variety of banding patterns, shared sequence homology. The only exception was strain RC1 which showed no homology to the dsRNAs found in hypovirulent strains from Michigan, other North American locations, or Europe.

Since most known mycoviruses have dsRNA as their genome, attempts were made to look for viral particles in C. parasitica. In the strains of C. parasitica studied to date, none of these dsRNA have been shown to be encapsidated within protein coats, unlike dsRNA of other filamentous fungi. Double-stranded RNA from the most thoroughly studied hypovirulent strain, EP113, is thought to be packaged within fungal vesicles (Hansen et al, 1985). Electron microscopic studies indicated that these vesicles are associated with the endoplasmic reticulum (Newhouse et al, 1983). Using this strain, Dodds (1980) isolated two fractions of membrane-bound vesicles, which differ in their density. Hansen et al.(1985) determined that one of the fractions contains most of the dsRNA. Although dsRNA of C. parasitica is not encapsidated within viral particles, the vesicles associated with the dsRNA appear to have an RNA polymerase associated with them. The vesicles containing greater amounts of dsRNA are able to incorporate nucleotide triphosphate into trichloroacetic acid (TCA) precipitable products. No detectable polymerase activity was found in the fraction containing little dsRNA and the similar vesicles from strains that do not have dsRNA (Hansen et al, 1985).

The composition of fungal vesicles is similar in both hypovirulent and virulent strains that do not contain dsRNA. The vesicles from the hypovirulent strain are much higher in lipid components and lower in protein content than those from the virulent strain. The protein composition is too low to account for the presence of a capsid. The neutral sugars that are present (arabinose, mannose, galactose, glucose) in these particles are also present in the fungal cell walls. This suggests that these vesicles are of fungal origin and are probably involved in cell wall synthesis (Hansen et al, 1985).

Structural properties of hypovirulence-associated dsRNAs are most studied in GH2, a North American strain and EP713, a European strain. These two dsRNA genomes reveal some common structural properties. Double-stranded RNA in both strains consist of multiple species, consisting of a large species (L-RNA) and other smaller ones. GH2 has two smaller molecules, namely M-RNA and S-RNA and EP713 has a number of medium and small dsRNAs. Hybridization studies and sequence analysis of some of the dsRNA shows that the smaller dsRNAs are derived by internal deletion of the L-RNA (Shapira et al, 1991b), with the exception of the GH2 S-RNA species. This was concluded because of the fact that the smaller dsRNA species have the same terminal sequence as the L-RNA. Double-stranded RNA of GH2 and EP713 contain a stretch of polyadenylic acid (poly A) at the 3'-terminus which base pairs with a stretch of polyuridinic acid (poly U) at the 5'-terminus of the complementary strand. This end is referred to as the homopolymer terminus and the other terminus is referred to as the heteropolymer end (Hiremath et al, 1986, Tartaglia et al, 1986, Nuss and Koltin, 1990).

Recent reports (Choi et al, 1991, and Shapira et al, 1991A) have revealed the complete nucleotide sequence and the genetic organization of the largest dsRNA (L-dsRNA) of hypovirulent strain EP713. L-dsRNA consists of 12,712 bp excluding the poly (A): poly (U)

homopolymer domain. The strand which terminates with a 3' poly (A) is referred to as the positive strand and contains two open reading frames, designated as ORF A and ORF B. ORF A encodes two polypeptides, p29 and p40, which are released from polyprotein, p69 (Choi et al, 1991). The cleavage is autocatalytic and is mediated by p29. The ORF A/ORF B junction consists of the sequence 5'-UAAUG-3', where the UAA of the pentanucleotide serves as the termination codon of the ORF A, whereas the AUG portion serves as the 5' proximal initiation codon of ORF B. This situation is similar to a pentanucleotide sequence observed in influenza virus RNA segment 7. ORF B also appears to be involved in an autocatalytic proteolysis event and encodes a 48 kd polypeptide, p48. Both polypeptides, p29 and p48, are presumably involved in catalyzing additional steps in the processing of the predicted ORF B encoded polyprotein. A helicase motif has been identified within the carboxyl terminal domain of ORF B (Shapira et al, 1991A).

Another report by Shapira et al. (1991B) strengthened the evidence that the M-dsRNA (8-10 kb) and S-dsRNA (0.6-1.7 kb) of EP713 are derived from the L-dsRNA by internal deletion. Sequence analysis of cloned cDNA copies of M-dsRNA and S-dsRNA species confirmed that these elements retained both heteropolymor and homopolymer domains found in L-dsRNA.

In spite of these recent developments in characterization of dsRNA molecules, it is still not clear how dsRNA affects the virulence of the fungus. A transformation system for C. parasitica has been developed (Churchill et al. 1990) and the transformation and expression of the cDNA clones of dsRNA into isogenic virulent strains may help in better understanding the role of dsRNA in hypovirulence (Hillman, 1990).

Even though dsRNA is correlated with the hypovirulence of C. parasitica in many strains, there are exceptions. In Michigan, hypovirulent strains which do not harbor detectable

amounts of dsRNA have been isolated from Crystal Lake, (Frankfort) and Kellogg Forest, (Augusta). Genetic studies on one of these dsRNA-free strains, CL25, showed that this type of hypovirulence is carried in 20% of the total conidia, is maternally inherited and can be cytoplasmically transferred to other virulent strains via hyphal anastomosis (Fulbright, 1985). This suggests that there is a cytoplasmic "virulence inhibition factor" other than dsRNA involved in the dsRNA-free type of hypovirulence since dsRNA is not maternally inherited. There are several reports on the debilitation of fungi which are associated with mitochondrial plasmids. Some of those model systems are described below.

Mitochondrial plasmids of filamentous fungi

Mitochondrial plasmids of filamentous fungi have been classified into two groups. "True" mitochondrial plasmids are defined as DNA or RNA species that are present in mitochondria, but are not derived from the mtDNA (Lambowitz et al 1986). True mitochondrial plasmids appear to have neither a positive nor a negative effect on the fungus. Examples of such plasmid are seen in mitochondria of higher plants, mushrooms, and several filamentous fungi, including Neurospora and Claviceps purpurea. "Defective mitochondrial DNAs" are circular DNAs found in the mitochondria and they are derived from the mitochondrial genome by excision and amplification. They are often suppressive relative to wild-type mitochondrial DNA (mtDNA) and accumulation of defective mtDNA is usually associated with a respiration-deficient phenotype and vegetative senescence. The classical examples are petite mutants in yeast (Locker et al, 1979), stopper mutants of Neurospora (Bertrand et al. 1980), ragged mutants of Aspergillus (Lazarus and Kuntzel, 1981) and senescence in Podospora (Wright 1982).

Mitochondrial plasmids of Neurospora

Mitochondrial plasmids were first discovered and characterized in Neurospora (Collins et al. 1981). These are true mitochondrial plasmids since they are not derived from the standard mtDNA. Three different circular plasmids, termed Fiji, LaBelle and Mauriceville, are found in different strains of Neurospora and they belong to three different homology groups.

Mauriceville plasmid contains a long ORF and encodes a hydrophilic polypeptide of 710 amino acids or one or more smaller polypeptides beginning at internal AUG codons. These polypeptides show homology to the proteins encoded by group II introns. The long ORF of the plasmid uses distinctive codons that are characteristic of ORFs in introns. These characteristics suggest that this plasmid is related to introns found in mtDNA. Others suggest that Mauriceville is related to transposable elements. Michael and Lang (1985) showed that proteins encoded by the Mauriceville plasmid contain short blocks of conserved amino acids that are characteristic of reverse transcriptase and related proteins. Characteristics of the plasmid suggest that it may belong to a class of mobile elements that are related to introns of mtDNA.

Unlike Mauriceville, LaBelle plasmid does not produce any discrete transcript. The ORF of LaBelle has no homology to reverse transcriptase. These findings suggest that LaBelle plasmids belong to a different class of intron related genetic elements than does the Mauriceville plasmid.

Several field isolates of Neurospora from Hawaii and India contain linear mitochondrial plasmids termed kalilo and maranhar respectively (Bertrand et al. 1989; Court et al.1991). These linear plasmids induce senescence and are absent in long-lived strains. The kalilo plasmid is about 9 kb long, whereas the maranhar plasmid has a length of 7.2 kb

(Bertrand and Griffith, 1989). Both these plasmids are present in high copy numbers in presenescent, juvenile as well as the senescent hyphae of the senescence-prone strains. Kalilo and maranhar plasmids have dissimilar nucleotide sequences, but they have very similar overall characteristics. Both plasmids have covalently-linked 5'-terminal proteins (Vierula et al 1990) and long inverted terminal repeats. DNA of both plasmids contain two long open reading frames (Bertrand and Griffith, 1989), one coding for an RNA polymerase and the other coding for a DNA polymerase. The two open reading frames are located on two opposite strands of the DNA in both the plasmids. These plasmids cause senescence by inserting into the mitochondrial genome which leads to the progressive accumulation of mutant forms of mtDNA. This ultimately results in respiratory deficiencies.

Mitochondrial plasmids of Podospora anserina

During senescence in Podospora, specific regions of the mitochondrial genome excise and replicate as autonomous plasmids. One plasmid, termed α senDNA is found more frequently but others are also observed (β, ϵ and θ senDNA). In senescing cultures, the 94 kb mitochondrial DNA was shown to be replaced by multimeric sets of small circular DNA (Cummings, 1979). These small circular molecules consist of head-to-tail tandem arrays of specific regions of the mitochondrial genome (Jamet-Vierney et al.1980). α senDNA is 2.6 kb long and the DNA sequence analysis shows that this 2.6 kb fragment is a complete group II intron of the cytochrome oxidase subunit 1 (COI) gene (Cummings and Wright 1983). Excision of α senDNA occurs precisely at the exon-intron junction within the mosaic gene encoding COI. Like other group II introns, α senDNA codes for amino acids sequences that have significant homology with retroviral reverse transcriptase (Matsura et al 1986). β senDNA and ϵ senDNA are 9.8 kb and 5.5 kb long, respectively. No introns have been found in β senDNA whereas in θ senDNA three group I introns have been found.

Long-lived mutants in Podospora are defined as strains capable of growth renewal after one or more senescent stoppages. (Cummings, 1986). These mutants lack the region corresponding to α senDNA. Belcour and Vierney (1986) sequenced across the deleted regions of α senDNA and found that α senDNA was not precisely excised. Long lived mutants reveal the presence of a novel family of small, autonomously replicating elements termed sMt-1, s-Mt2 and s-Mt3. DNA sequence analysis shows that s-Mt1 is 368 bp, s-Mt2 is 114 bp and s-Mt3 is 67 bp. The s-Mt2 and s-Mt3 elements overlap and are completely contained within the s-Mt1 element. In early stages of growth, several of these long-lived strains contain α senDNA but none of the sMt plasmid. Later in the vegetative growth the sMt elements are observed. Gene rearrangement of the mitochondrial genome is seen as soon as the sMt plasmids appear. It is believed that the senDNA and the sMt elements play an antagonistic role in the determination of life span.

"Ragged" mutants of Aspergillus

Ragged mutants of Aspergillus contain head-to-tail repeats of plasmid like sequences which arise from different areas of the mtDNA. In one mutant strain (Rgd 1), the amplified mtDNA segment (rgd 1 DNA, monomeric length 0.9kb) maps downstream of the large subunit ribosomal gene whereas in other strains (Rgd 3), the amplified sequence (rgd 3-7 DNA) are located between genes coding for cytochrome b and ATPase subunit 6. These ragged mutants display a number of physiological defects, including cytochrome abnormalities and high levels of cyanide-insensitive respiration (Lazarus and Kuntzel, 1981).

Mitochondrial plasmids of Claviceps purpurea

Claviceps purpurea is a fungal phytopathogen of cereals and nonagricultural grasses. Mitochondrial plasmids were discovered and characterized in strain K1 from Germany and other strains. There are three main mitochondrial plasmids in Claviceps. They are termed pC1K1, pC1K2 and pC1K3 and are 6.7, 5.5 and 1.1 kb, respectively. The smaller plasmids are homologous to the larger plasmid. There is no homology between the plasmids and mtDNA. These plasmids are linear and contain terminal inverted repeats (TIRs). In at least two strains, plasmid-homologous segments can be found in the mtDNA. This is thought to be the result of integration events like those found in Neurospora with the kalilo and maranhar plasmids (Tudzynski and Esser 1986; Bertrand and Griffiths, 1989). In C. purpurea, there appears to be no deleterious effects on fungal metabolism of plasmid integration.

Mitochondrial Plasmids in Cochliobolus heterostrophus

Cochliobolus heterostrophus, a fungal phytopathogen of maize, has two mitochondrial plasmids called T40 and T21. They are both circular and exist as a series of head-to-tail multimers. The mitochondrial genome is not noticeably affected by the presence of the plasmid and the plasmid containing strain does not exhibit any distinct phenotype. Plasmids T40 and T21 have the monomenic sizes of 1.8 and 2.2 kb, respectively. They share 1.5 kb in common. Portions of both plasmids show high homology with the mitochondrial ATPase subunit 6 gene. The gene is present in its entirety on the T40 plasmid, whereas the first 130 nucleotides at the 5' end of the gene are missing from the T21 plasmid, suggesting that the presence of an intact copy of ATPase submit 6 gene in the plasmid is not required either for the maintenance of the plasmid or the growth of strain T21.

Alternative oxidase pathway: cyanide-resistant respiration

It is apparent that the aberrant growth phenomena encountered among some filamentous fungi may be associated with grossly defective mtDNA. Physiological defects leading to abnormal growth of the fungus in culture appears to be the result of cytochrome abnormalities which are associated with high levels of cyanide-resistant respiration and defective mtDNA.

The presence of an alternate, cyanide-resistant pathway of mitochondrial respiration has been reported in filamentous fungi, yeast, green algae, and higher plants. The alternate oxidase pathway is of great interest in that energy is not generated in the form of ATP when the electrons flow through it. The potential energy of the system is lost as heat. The electron transport in these mitochondria is branched, with a classical cyanide-sensitive cytochrome pathway that terminates with cytochrome oxidase and a hydroxamic acid-sensitive pathway that terminates with an alternative oxidase. The alternative pathway was shown to branch from the cytochrome chain at the ubiquinone pool (Storey, 1976). In both higher plants and Neurospora mitochondria, a remarkable feature of the branch point is its ability to regulate electron flow so that the cytochrome system is used maximally and the alternate oxidase is used only to accommodate surplus electron flow (Lambowitz et al.1972, Edwards et al. 1974). Direct measurement of phosphate esterification in Neurospora shows that the alternate oxidase does not result in oxidative phosphorylation (Lambowitz et al. 1972), a feature common with alternative pathways of higher plants and yeast mitochondria (Henry and Nyns, 1975).

Poky (Mi-1) is perhaps the best known of the cytochrome deficient mutants of Neurospora. In wild type strains, more than 90% of total respiration is mediated by a conventional cytochrome chain (sensitive to antimycin and cyanide). Poky mutants use both

the cytochrome chain and an alternate oxidase (resistant to antimycin and cyanide, but sensitive to salicyl hydroxamic acid). The alternate oxidase pathway accounts for as much as 70% of the respiration in poky mitochondria.

A comparative spectrophotometric study of the electron transport system in mitochondria from wild-type and poky strains of Neurospora showed striking differences in the concentrations of electron carriers. Poky mitochondria contain flavoproteins and cytochrome c at concentration that are 30% higher than that of wild type mitochondria, but poky mitochondria are grossly deficient in b and a type cytochromes. The increased concentration of flavoproteins in poky mitochondria led to speculation that the alternate oxidase might be a flavoprotein (Lambowitz et al. 1972).

In wild-type Neurospora, alternate oxidase is not present constitutively but can be induced by a variety of treatments that share the capacity to impair the cytochrome system. The agents include antimycin, cyanide, oligomycin, CCCP, chloramphenicol (a specific inhibitor of mitochondrial protein synthesis) and ethidium bromide (an inhibitor of mitochondrial DNA transcription). Using actinomycin D, an inhibitor of nuclear transcription, Edwards et al. (1973) reported that wild-type Neurospora strains require de novo expression of nuclear genes. These data are compatible with the model proposed by Barath and Kuntzel (1972) for the regulation of nuclear genes specifying the mitochondrial genetic apparatus of Neurospora. This model proposes the synthesis of mitochondrially coded repressor proteins on mitochondrial ribosomes. These proteins are then transported to the nuclear genes that they repress. As long as mitochondrial protein synthesis continues to function normally, repressor proteins are synthesized and the nuclear genes are repressed. Treatment with chloramphenicol results in inhibition of mitochondrial protein synthesis with the resultant depression of the repressed genes.

Edwards and co-workers studied six antimycin-sensitive (ANT) mutants which could not synthesize functional alternate oxidase and found them to fall into two complementation groups, alx-1 and alx-2 (Edwards et al. 1976). Bertrand et al. (1983) have characterized 27 antimycin-sensitive mutants of Neurospora which are unable to induce alternate oxidase activity. These mutants fall into two complementation groups, aod-1 and aod-2. In response to the different inducing conditions, the wild type strain and 19 out of 20 aod-1 mutants were found to accumulate a polypeptide that was tentatively identified as being associated with alternate oxidase. The remaining aod-1 mutants and all four aod-2 mutants that were tested did not accumulate this polypeptide. Based on those findings, Bertrand et al (1983) hypothesized that aod-1 is the structural gene for alternate oxidase and that the aod-2 gene encodes a component that is required for induction of alternate oxidase activity.

Elthon and McIntosh (1987) have raised polyclonal antibodies to the solubilized and partially purified alternate oxidase from the thermogenic Voodoo lily, Sauratum guttatum, the antiserum cross reacts with 37, 36 and 35 KD polypeptides. Monoclonal antibodies have now have been prepared to these proteins and designated as AOA (binds to all three proteins of the alternate oxidase cluster), AOU (binds to 37 KD protein) and AOL (binds to the lower 36 and 37 KD proteins). These polypeptides appear to be remarkably conserved and the antibodies cross-react with similar proteins in nonthermogenic plant species and also Neurospora which makes these antibodies useful in identifying alternate oxidase proteins in other systems.

Using the monoclonal antibody (AOA) and two alternate oxidase mutants (aod-1,aod-2) described above, Lambowitz et al.(1989) found that aod-1 mutants produced inactive polypeptides whereas aod-2 mutants gene failed to produce these polypeptides. These findings support the previous hypothesis of Bertrand (1983) that aod-1 is a structural gene for the

alternate oxidase and that the aod-2 gene encodes a component that is required for induction of alternate oxidase activity. This work also shows that the induction of alternate oxidase in Neurospora results from the synthesis of alternate oxidase polypeptides and not from the activation of pre-existing polypeptides.

The physiological role of alternate oxidase is best understood in thermogenic higher plants, most of which are members of the Araceae. In the flowers or inflorescence of these plants, heat produced by way of electron flow through the alternate oxidase pathway is often used to volatilize insect attractants facilitating pollination. In Neurospora, cells that predominantly use this pathway, (poky, and wild type grown in the presence of antimycin) have conspicuously slow growth rates, which seem to be related to the residual activity of the cytochrome system rather than to the level of the alternate oxidase (Lambowitz and Slayman. 1972). For most plants and fungi, the physiological role of alternate oxidase is obscure. One hypothesis is that it serves as a method to rid the cells of excess carbohydrates (Lambowitz et al. 1978, Lambers. 1982). Another possibility is that the alternate oxidase defends the fungus or plant against respiratory inhibitors produced by competing organisms in the environment (Lambowitz and Zannoni, 1978). The hypothesis is suggested by studies of the fungus Stremphylium loti (Rissler and Miller,1977), which as a pathogen of cyanogenic pants is likely to encounter cyanide in its natural environment. Rissler and Miller noted that induction of alternate oxidase in this organism following the addition of cyanide is correlated with the induction of formamide hydrolase activity. This enzyme converts cyanide to nontoxic formamide and is thought to be partially responsible for the tolerance of S. loti to high concentrations of cyanide in vitro. Fungi, bacteria and higher plants have the ability to produce antibiotics and toxins. The presence of an insensitive alternate oxidase pathway may attribute to an important selective advantage in such an environment.

My work in this thesis involves several recently discovered hypovirulent isolates of C. parasitica that do not contain detectable amounts of dsRNA. Genetic studies with one of these dsRNA-free hypovirulent strains, CL25, from Crystal Lake, MI. suggests that the factor(s) responsible for the dsRNA-free hypovirulence phenotype is cytoplasmic in nature. In this thesis I have asked the following questions to help understand the basis of this novel form of hypovirulence.

* Are plasmids involved in the hypovirulence of C. parasitica?

* Are mitochondria associated with the dsRNA-free hypovirulence phenotype and if so, can they be transferred to other virulent strains via hyphal fusion?

* Are respiratory defects partially or totally responsible for hypovirulence of C. parasitica?

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CHAPTER-1

CRYPTIC MITOCHONDRIAL PLASMIDS IN *CRYPHONECTRIA PARASITICA*.

ABSTRACT

Hypovirulent strains of *Cryphonectria parasitica* have been associated with the presence of cytoplasmically transmissible double-stranded RNA (dsRNA). In Michigan, several hypovirulent strains of *C. parasitica* have been discovered that do not harbor detectable levels of dsRNA. Genetic studies with a dsRNA-free hypovirulent isolate, CL25, suggests that there is a transmissible, maternally inherited cytoplasmic "virulence inhibition factor" other than dsRNA that is responsible for the hypovirulence phenotype of these dsRNA-free strains. A study of cytoplasmic agents revealed the presence of two low copy number plasmids in the mitochondria. These plasmids are about 6 and 10 kilobase pairs (kb) in size. Hybridization studies with a cloned fragment of the 6 kb plasmid, indicated the presence of this plasmid in all virulent and hypovirulent *C. parasitica* strains screened regardless of geographical origin. The cloned plasmid DNA also hybridized to plasmid-like elements of a different size in *Endothia gyrosa*. This result suggests that the 6 kb plasmid may not play a direct role in the hypovirulence phenotype of CL25 and other strains. The cloned fragment of the plasmid does not hybridize with the mitochondrial genome, and the function of the plasmid remains cryptic.

INTRODUCTION

The fungal phytopathogen Cryphonectria (=Endothia) parasitica has eliminated the American chestnut [Castanea dentata (Marsh) Borkh] as a major tree species in the Eastern deciduous forests of North America. Chestnut blight also invaded European chestnut forests and orchards (Castanea sativa Mill.) and caused considerable damage. It is thought that this fungus was introduced into North America on nursery stock of oriental chestnuts and the fungus spread very quickly to the susceptible American chestnut trees. Hypovirulent strains have been isolated from recovering chestnut trees in Europe and North America where chestnut trees survive infection. Hypovirulent strains are reduced in virulence, produce fewer conidia and exhibit unusual culture morphology. Hypovirulence in C. parasitica can be transferred to other virulent strains via hyphal anastomosis and has been correlated with the presence of dsRNA.

One fungal strain from Crystal Lake, Michigan, has many characteristics of dsRNA-associated hypovirulence but harbors no detectable dsRNA. Genetic studies with CL25 reveal that this type of hypovirulence is carried by 20% of the conidia (asexual spores), is maternally inherited, and is cytoplasmically transferred to other virulent strains via hyphal anastomosis (Fulbright, 1985). This suggests that there is a "virulence inhibition factor" (VIF) other than dsRNA responsible for the dsRNA-free hypovirulent phenotype of CL25.

Few studies have made comparisons between the hypovirulence phenotype and senescence, a phenomenon encountered in filamentous fungi where cellular death occurs after prolonged asexual propagation (Bertrand, 1983). The symptoms of senescence include female sterility, sterility of conidia, abnormal growth morphology and respiratory deficiencies. Senescence is exhibited by several filamentous fungi such as "stopper" mutants of N. crassa, where the fungus has an unusual "stop-start" growth phenotype on solid media (Bertrand et

al. 1980); Aspergillus, which exhibits a characteristic "ragged" appearance due to vegetative death of the hyphal tips (Lazarus et al, 1980); and, Podospora, where vegetative death of hyphal tips occur (Smith and Rubenstein, 1973). In all cases of senescence described above, there is evidence of the involvement of cytoplasmic factors within the mitochondria. Mitochondria of senescing fungi invariably accumulate a diversity of plasmids which are usually deletions of large segments of mtDNA resulting from intramolecular recombination as seen in "stopper" mutants of N. crassa (Bertrand, 1983; Gross et al. 1984) and the ragged mutants of Aspergillus (Lazarus and Kuntzel, 1981). In P. anserina, plasmids result from excision of small fragments of mtDNA rather than intramolecular recombination (Cummings, 1979). Based on the association of mitochondrial plasmids with senescence and similar features shared by both hypovirulence and senescence, I decided to examine the possible involvement of mitochondrial plasmids in the hypovirulence phenotype of C. parasitica.

Here, I present evidence of the existence of at least two plasmids in the mitochondria and show that at least one of these elements may not be directly associated with hypovirulence of C. parasitica.

MATERIALS AND METHODS

Fungal strains and growth conditions

Cryphonectria parasitica strains used in this study are listed in Table-1.1. Endothia gyrosa and Colletotrichum lagenarium were used as controls (*Table-1.1). Cultures of C. parasitica were maintained on potato dextrose agar (PDA, Difco) at 20°C under fluorescent light. Cultures used for mitochondrial DNA isolation were grown for 10-14 days in the liquid medium of Puhalla and Anagnostakis (1971) modified by the omission of glucose.

TABLE 1.1- C. parasitica strains used in the plasmid screening study.

Strain	Virulence	Description
CL25	HV	Isolated from a non lethal canker from Crystal Lake, MI.; no dsRNA.
CL1-16	V	Single spore isolate of a virulent strain from Crystal Lake, MI.
EP155	V	Virulent strain from Connecticut.
GHU4	HV	A dsRNA containing hypovirulent strain from Grand Haven, MI.
TR48	HV	Strain from Italy Europe; dsRNA present.
NCF-B	V	Strain from Accokeek, MD.
JG	HV	Strain from Judy Gap, WV, dsRNA present.

HV= Hypovirulent, V= Virulent.

*Colletrichum lagenarium and Endothia gyrosa cultures were kindly donated to us by Dr. Alice Bonnen and Mark Double, respectively.

Mitochondrial DNA Isolation

Mitochondrial DNA was isolated following the procedure of Mackenzie et al (1988) with several modifications. Twelve-day-old cultures of C. parasitica were strained through Miracloth (Calbiochem) and pressed dry. Twenty to twenty-five grams (pressed weight) of fungus was ground in liquid nitrogen with glass beads using a chilled mortar and pestle. Ground mycelia were stirred in 400 mls of cold buffer A (0.5M sucrose, 0.05M Tris, 0.005M EDTA, 0.1% BSA, pH 7.5 and 0.005M mercaptoethanol added just before use) for 10 min. The homogenate was filtered through two layers of Miracloth and centrifuged for 15 min at 1,000Xg to pellet nuclei. The resulting supernatant was centrifuged at 15,900Xg for 20 min. The pellet was resuspended with a paint brush in buffer G (0.03M sucrose, 0.05M Tris, pH 7.5) using 10 ml per 20 g starting material. After centrifuging for 10 min at 1,000Xg, the supernatant was brought to 10 mM $MgCl_2$ and 20 µg DNase (Sigma, Type IV)/ml or 200 Kunitz units/ml, and incubated with gentle mixing at 4°C for 60 min. The suspension was underlaid with 20 ml shelf buffer (0.6M sucrose, 0.01M Tris, 0.02M Na_2EDTA, pH 7.5) per 5 ml and centrifuged for 25 min at 12,000Xg. The pellet was resuspended with the shelf buffer (5 ml) and the mitochondria were pelleted by centrifuging for another 25 min at 15,900Xg. The mitochondria were then lysed in 5 ml lysis buffer (0.01M Tris, 0.05M EDTA, 0.1M Nacl, 1% SDS, pH 8.0)/20-25g of fungus and 100 µg proteinase K/ml at 65°C for 30 min. The protein-carbohydrate complex was precipitated by adding one third the volume 5M potassium-acetate, incubating on ice for one hour with periodic shaking, and centrifuging for 20 min at 25,000Xg. The supernatant was poured into a siliconized corex tube and the mtDNA precipitated with 1/20 volume of 5M ammonium acetate and 1/2 volume of ice cold isopropanol at -20° C.

The mtDNA was pelleted by centrifuging at 10,000Xg for 45 min. The pellets were air dried, dissolved in 0.7 ml T50E10 buffer (50mM Tris, 10mM EDTA, pH 8.0). The solution

was transferred to a microfuge tube and microfuged for 10 min. The supernatant was transferred to a clean tube and the mtDNA was precipitated as above with ammonium acetate and isopropanol. The mtDNA was washed twice with 70% ethanol, vacuum dried for 10-15 min, and allowed to dissolve in TE (10mM Tris, 1mM EDTA, pH 8.0) overnight at 4° C.

Electrophoresis of mitochondrial DNA

The mtDNA was electrophoresed in 0.7% agarose gels using TBE buffer (Maniatis et al, 1982). Molecular size markers were obtained by digesting Lambda DNA with HindIII. Gels were electrophoresed at room temperature for 8 hours at a constant voltage of 50-60 volts. The gels were stained with ethidium bromide (0.5 μg/ml) for 15-20 min and photographed. The mtDNA in the gel was transferred to nylon hybridization membranes (MSI, Westboro, MA.) as described by Maniatis et al (1982). The DNA was fixed on the membrane by UV crosslinking for 3-5 min.

Isolation and cloning of the plasmids

Mitochondrial DNA of CL25 was electrophoresed on an agarose gel under the same conditions as described above. After staining the gel with ethidium bromide, the agarose containing the two plasmid bands was cut out from several lanes and the DNA was electroeluted with an electroeluter using the procedure suggested by the manufacturer (Schleicher and Schuell). The buffer with the electroeluted DNA was precipitated overnight with 2 volumes of absolute EtOH and 1/10 volume of 3 M NaOAc at 0°C. The DNA was collected by spinning at 15K for 50 min.

The electroeluted DNA was digested with restriction enzyme BglII and was cloned into the BamH1 site of the polylinker of cloning vector pRL498 (Elhai and Wolk, 1988). The

boiling lysis miniprep method described by Maniatis et al (1982) was used to identify and prepare the resulting recombinant plasmid, pNK40.

DNA labelling and hybridization

The recombinant plasmid was digested with restriction enzyme HindIII and electrophoresed in an agarose gel. The insert was cut out of the gel, electroeluted and used for probes after radiolabelling. Probes were radiolabelled using the random primer method of Feinberg and Volgenstein (1983).

RESULTS AND DISCUSSION

The mitochondria of filamentous fungi contain a wide range of plasmids and plasmid-like elements. These plasmids show many different characteristics in a wide range of fungal species. Many of these plasmids do not have a phenotype or a physiological role associated with them. Examples of these are the Mauriceville, Fiji and LaBelle plasmids of Neurospora (Collins et al, 1981); T40, T21 of Cochliobolus (Garber et al,1986); and pC1K1, pC1K2 and pC1K3 of Claviceps purpurea (Tudzynski et al, 1986). Other mitochondrial plasmids affect the mitochondrial genome, as seen with kalilo and maranhar plasmids of Neurospora (Bertrand et al. 1986, Court et al. 1991). The plasmids of Podospora anserina are believed to be derived from the mtDNA by deletion and amplification. In both events, the mitochondria become progressively deficient in cytochrome aa_3 and b, and begin to accumulate excess cytochrome c and flavoprotein (Lambowitz et al. 1972, Tissiers et al. 1953, Lambowitz and Zannoni. 1978, Bertrand et al. 1976). It is thought that these changes lead to senescence in those fungal strains.

When undigested mtDNA of C. parasitica is electrophoresed in an agarose gel, two DNA bands of approximately 6 and 10 kb are seen in addition to the mitochondrial genome

(Fig 1.1). These plasmids are present in low copy number and are often difficult to visualize on ethidium bromide stained gels. Restriction digest of these plasmids after electroelution from the agarose gel showed that the 10 kb plasmid band can be digested with restriction enzyme EcoR1 whereas the 6 kb plasmid cannot be digested with EcoR1 (Fig 1.2). These results indicated that the two plasmids are in fact two different plasmids and not different forms of the same plasmid. This was again confirmed with DNA hybridization studies (Fig 1.3). A 2.5 kb fragment clone, pNK40, of the 6 kb plasmid was used to screen several virulent and hypovirulent strains from different geographical locations. This clone hybridized to all the C. parasitica strains assayed. The clone pNK40 frequently hybridized to three different bands which suggested that the 6 kb plasmid may be a circular plasmid and that the three bands corresponded to different forms of the same plasmid. There was also hybridization to larger DNA bands which are probably multimeric forms of the plasmid. This clone did not hybridize to the 10 kb plasmid, substantiating the earlier interpretation that these plasmids are different. Hybridization of pNK40 also occurred to plasmid-like elements in Endothia gyrosa (Fig 1.3-B, lane 9). This is a species that is believed to be related to C. parasitica (previously known as Endothia parasitica). Not only was the plasmid-like band in E. gyrosa of a different size, but the E. gyrosa strain also had a different mitochondrial DNA banding pattern (data not shown). Mitochondrial DNA of Colletotrichum lagenarium was used as a negative control (Fig 1.3-B, lane 8).

Since the 6 kb plasmid is present in all hypovirulent and virulent strains screened, the direct involvement of this plasmid in hypovirulence of C. parasitica is doubtful. An indirect role of the 6 and 10 kb plasmids in the hypovirulence phenotype can not yet be ruled out as they might be involved in mtDNA aberration.

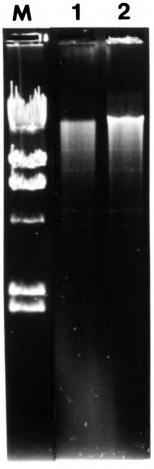

Fig 1.1- Intact mtDNA of isogenic strains of C. parasitica.
Lane 1- CL25#9 (Hypovirulent)
Lane 2- CL25#4 (virulent)
M- λ DNA digested with HindIII used as molecular size marker. The sizes of the bands from top to bottom are 23.13, 9.41, 6.55, 4.36, 2.32, 2.02 kilo bases, respectively.

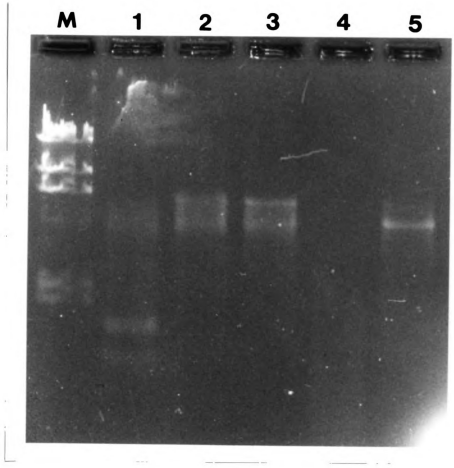

Fig 1.2- Mitochondrial plasmids digested with different enzymes.
Lane 1- BglII, 2- BamH1, 3- HindIII, 4- Sau3A, 5- EcoR1.
M- λ DNA digested with HindIII used as molecular size marker. The sizes of the bands from top to bottom are 23.13, 9.41, 6.55, 4.36, 2.32, 2.02 kilo bases, respectively.

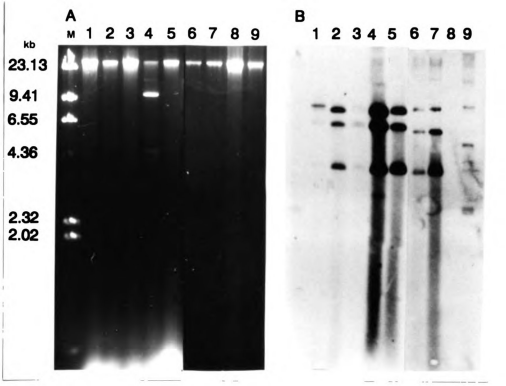

Fig 1.3- Intact mtDNA of different isolates of C. parasitica, Endothia gyrosa and Colletotrichum lagenarium.
Lane 1- GHU4, 2- JG, 3- TR48, 4- NCF-B, 5- EP155, 6- CL1-16, 7- CL25,
8- C. lagenarium, 9- E. gyrosa.
A- Ethidium bromide stained gel, M- λ DNA digested with HindIII used as molecular size marker.
B- Southern blot hybridized with a clone if a fragment of 6kb plasmid.

We did not observe the accumulation of a great diversity of plasmids in the dsRNA-free hypovirulence strains as has been observed in senescing strains of other fungal species. However, one strain of C. parasitica, NCF, showed the presence of unique very abundant DNA band other than the mitochondrial genome and the 6 and 10 kb plasmids observed in other strains (Fig 1.3-A, lane 4).

The 6 kb plasmid does not have any homology with the mitochondrial genome. Therefore it can be categorized as a "true mitochondrial plasmid", as defined by Lambowitz et al (1986). Like many other mitochondrial plasmids in other fungal species, no function has been attributed to this plasmid in C. parasitica.

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CHAPTER-2

TRANSFER OF MITOCHONDRIA DURING HYPHAL ANASTOMOSIS IN CRYPHONECTRIA PARASITICA.

ABSTRACT

Hypovirulent strains of the chestnut blight fungal phytopathogen, Cryphonectria parasitica have been shown to be associated with the presence of transmissible double-stranded RNA (dsRNA). Recently, hypovirulent strains have been isolated which do not harbor detectable dsRNA. The dsRNA-free hypovirulence phenotype is maternally inherited and transmissible to other C. parasitica strains during hyphal fusion indicating that the dsRNA-free hypovirulence phenotype is cytoplasmic in nature. Therefore, the involvement of mitochondria in dsRNA-free hypovirulence was investigated. To determine whether or not mitochondria can transfer during hyphal anastomosis, both genetic and molecular mitochondrial markers were constructed. First, a chloramphenicol resistant variant was isolated from the dsRNA-free hypovirulent strain CL25. The resistance phenotype was shown to be maternally inherited, indicating that the resistance was conferred by a mitochondrial gene. Secondly, a restriction fragment length polymorphism of 2.5 kb was found that was unique to CL25 mitochondrial DNA (mtDNA). The naturally occurring light pigmentation phenotype of CL25 was used as a nuclear genetic marker. Vegetative pairings were made between the light pigmented, chloramphenicol-resistant hypovirulent strain of CL25 and the

virulent, orange pigmented, chloramphenicol-sensitive strain, 4-C. After vegetative fusion, an orange pigmented subculture removed from the 4-C side of the pairing was shown to be chloramphenicol resistant and it contained the mitochondrial RFLP of both strains. The subculture containing mtDNA from both 4-C and CL25 produced only single-conidial isolates with orange pigmentation containing either one or the other mtDNA, indicating that the subculture was not contaminated by a hyphae or nuclei from CL25. In this chapter, I report on the use of nuclear and mitochondrial markers to provide evidence that mitochondria can transfer between strains of C. parasitica during hyphal anastomosis. This finding links the dsRNA-free hypovirulence phenotype of C. parasitica with its mitochondrial genome.

INTRODUCTION

Cryphonectria (= Endothia) parasitica (Murr.)Barr, the causal agent of chestnut blight was responsible for the demise of the once prevalent American chestnut (Castanea dentata [Marsh] Borkh) in North America and European chestnut (C. sativa Mill) in Europe. Isolates with reduced virulence (hypovirulent) have been found in Europe and North America where chestnut trees survive infection. Well-characterized hypovirulent strains of C. parasitica have been found to be infected with cytoplasmically transmissible double-stranded RNA (dsRNA) molecules which appear to be correlated with changes in virulence, growth rate, sporulation and/or culture morphology.

One strain of C. parasitica, CL25 from northern (Crystal Lake) Michigan, has all the characteristics of dsRNA-associated hypovirulence but harbors no detectable dsRNA (Fulbright, 1985). We have used 30 times the quantity of mycelium of CL25 for isolation of dsRNA, and still have been unable to detect dsRNA in this strain (unpublished data). Genetic studies of CL25 showed that hypovirulent and virulent phenotypes segregate in the

conidia of CL25, with hypovirulence being found in only 20% of the conidia. Furthermore, the hypovirulence phenotype is maternally-inherited and cytoplasmically-transferred via hyphal anastomosis (Fulbright, 1985). These studies suggest that there is a cytoplasmic "virulence inhibition factor" (VIF) other than dsRNA involved in the hypovirulence phenotype, since dsRNA-associated hypovirulence has not been found to be maternally inherited. Therefore, we initiated a study on the possible involvement of the mitochondrial genome in the dsRNA-free hypovirulence phenotype. If mitochondria are involved in dsRNA-free hypovirulence, mitochondria or genetic factors associated with mtDNA would have to transfer to other strains, along with the hypovirulence trait.

Since hypovirulence is a cytoplasmically transmissible trait, hyphal fusion must occur in order for the determinants for hypovirulence to be transferred (Anagnostakis, 1984). It has been demonstrated that dsRNA can successfully transfer between the strains that are vegetatively compatible and to some extent between vegetatively incompatible strains. The ability of dsRNA to spread between strains of different vegetative compatibility (vc) groups depends on the number of vc loci at which the groups differ (Anagnostakis, 1984). Information regarding the movement of mitochondria during hyphal fusion of compatible as well as between incompatible strains is limited. Some reports indicate that mitochondria do not move during hyphal fusion in filamentous fungi (Brasier, 1986; Casselton and Economou, 1985). Gobbi et al. (1990) reported the absence of mitochondrial transfer in C. parasitica, but pointed out that low levels of mitochondrial transmission could have evaded their detection. These reports on apparent lack of mitochondrial transfer during vegetative fusion contrast with the reports on mitochondrial transmission in other filamentous fungi. In the Basidiomycete, Coprinus cinereus, recombination of the mitochondrial genome has been shown to occur in the region of hyphal anastomosis (Baptista-Ferreira et al, 1983). In

Schizophyllum commune, another Basidiomycete, transfer of mitochondria was visualized by means of phase-contrast microscopy (Watrud and Ellingboe, 1973). Collins and Seville (1990) have provided evidence for the transfer of the mitochondrial chromosome and plasmids during unstable vegetative fusion in Neurospora. In similar studies by Griffith et al (1990), the transmission of a mitochondrial plasmid in a transient fusion between N. intermedia and N. crassa cells was reported. Due to the conflicting reports about mtDNA transfer in fungi, we decided to reexamine the question of mitochondrial transmission in C. parasitica. Using mitochondrial and nuclear markers, we present evidence that mitochondria in C. parasitica can transfer between strains during hyphal anastomosis.

MATERIALS AND METHODS

Fungal strains and growth conditions

Strains used in this study are listed in Table 2.1. CL25 is a bark isolate of C. parasitica from Crystal Lake, Michigan (Fulbright, 1985). This is a light pigmented hypovirulent isolate without detectable amounts of dsRNA. Strain 4-C is a dark pigmented virulent isolate without dsRNA. Single conidial isolates from these cultures consistently maintained the pigmentation trait of the parent.

A chloramphenicol resistant strain was isolated using the procedure of Brasier and Kirk (1986). A range of eight concentrations of chloramphenicol (2-10 mg/ml) in potato dextrose agar (PDA) were inoculated with CL25 to determine the inhibitory concentrations of chloramphenicol suitable for selection of mutants. Marginal outgrowths from mycelium growing on 8 mg chloramphenicol/ml were subcultured on PDA plates supplemented with 8 mg chloramphenicol/ml.

TABLE 2.1. Strains of Cryphonectria parasitica used in the study of mitochondrial transfer during hyphal anastomosis.

Strain	Virulence	Description
CL25	HV	Isolated from a healing canker at Crystal Lake, MI.
CL25 cap^R	HV	A light pigmented chloramphenicol resistant strain of CL25.
4-C	V	An orange pigmented strain derived from GHU4 by curing of dsRNA (Fulbright, 1984).

HV= Hypovirulent, V= Virulent
CL25- a hypovirulent strain without any detectable amounts of dsRNA.
GHU4- a hypovirulent strain with dsRNA.

Cultures used in this study were maintained on PDA at 20°C under fluorescent light. Cultures for mtDNA isolation were grown for 10-14 days in the liquid medium of Puhalla and Anagnostakis (1971) modified by the omission of glucose. Liquid medium was amended by the addition of 2-2.5 mg chloramphenicol/ml as appropriate. The chloramphenicol resistant isolates were maintained on plates with 8 mg chloramphenicol/ml.

Sexual crosses

Sexual crosses were performed between fungal strains using the procedure of Anagnostakis (1979). A chloramphenicol-resistant strain of CL25 and a chloramphenicol-sensitive virulent strain were crossed by pouring 4% water agar around an autoclaved chestnut twig in a petri dish (Difco). Mycelial plugs of the maternal strain were placed on the agar surface on either side of chestnut wood and the fungal strain was allowed to grow on the wood for about 20 days. When stromata formed on the bark, a conidial suspension of the paternal parent, collected from a culture growing on PDA, was added to the plate and shaken to cover the stroma. Perithecial necks were visible above the stromata 7-8 weeks later. Perithecia were removed and ascospores collected and grown on PDA and PDA supplemented with 8 mg chloramphenicol/ml.

Single-conidial isolation

To harvest conidia, 1 ml sterile distilled water was poured on the mycelial mat of 10-day-old cultures grown on PDA. The plate was shaken gently to free the conidia from the mat. The conidial suspension was collected in a sterile tube and 10-fold serial dilutions (up to five) were made in distilled water. One hundred µl of each dilution was placed on PDA plates and spread with a sterile glass rod. These plates were placed under fluorescent lights

for 1-2 days and germinating spores were visualized with a dissecting microscope. Single-conidial isolates were obtained by transferring small pieces of agar with the germinating spore to PDA plates.

Pathogenicity test

Fungal isolates were tested for virulence by placing 7 mm mycelial plugs from 10-day-old PDA cultures into 9 x 7 mm-deep holes on the side of Golden Delicious apples (Fulbright, 1984). The inoculation holes were covered with tape to prevent desiccation and the apples were placed in open plastic bags for 3 weeks.

Mitochondrial DNA Isolation

Mitochondrial DNA was isolated following the procedure by Mackenzie et al (1988) with several modifications. Twelve-day-old cultures of C. parasitica were strained through Miracloth (Calbiochem) and pressed dry. Twenty to twenty-five grams (pressed weight) of fungus was ground in liquid nitrogen with glass beads using a chilled mortar and pestle. Ground mycelia were stirred in 400 mls of cold buffer A (0.5M sucrose, 0.05M Tris, 0.005M EDTA, 0.1% BSA, pH 7.5 and 0.005M mercaptoethanol added just before use) for 10 min. The homogenate was filtered through two layers of Miracloth and centrifuged for 15 min at 1,000Xg to pellet nuclei. The resulting supernatant was centrifuged at 15,900Xg for 20 min. The pellet was resuspended with a paint brush in 10 ml buffer G (0.03M sucrose, 0.05M Tris, pH 7.5) per 20 g starting material. After centrifuging for 10 min at 1,000Xg the supernatant was brought to 10 mM $MgCl_2$ and 20 µg DNase (Sigma; Type IV)/ml or 200 Kunitz units/ml, and incubated with gentle mixing at 4°C for 60 min. The suspension was underlaid with 20 ml shelf buffer (0.6M sucrose, 0.01M Tris, 0.02M Na_2EDTA, pH 7.5) per 5 ml and

centrifuged for 25 min at 12,000Xg. The pellet was resuspended with the shelf buffer (5ml) and the mitochondria were pelleted by centrifuging for another 25 min at 15,900Xg. The mitochondria were then lysed in 5 ml lysis buffer (0.01M Tris, 0.05M EDTA, 0.1M Nacl, 1% SDS, pH 8.0)/20-25g of fungus and 100 µg proteinase K/ml at 65°C for 30 min. The protein-carbohydrate complex was precipitated by adding one third the volume 5M potassium-acetate, incubating on ice for one hour with periodic shaking, and centrifuging for 20 min at 25,000Xg. The supernatant was poured into a siliconized corex tube and the mtDNA precipitated with 1/20 volume of 5M ammonium acetate and 1/2 volume of ice cold isopropanol at -20° C.

The mtDNA was pelleted by centrifuging at 10,000Xg for 45 min. The pellets were air dried, dissolved in 0.7 ml T50E10 buffer (50mM Tris, 10mM EDTA, pH 8.0). The solution was transferred to a microfuge tube and centrifuged for 10 min. The supernatant was transferred to a clean tube and the mtDNA was precipitated as above with ammonium acetate and isopropanol. The mtDNA was washed twice with 70% ethanol, vacuum dried for 10-15 min, and allowed to dissolve in TE (10mM Tris, 1mM EDTA, pH 8.0) overnight at 4° C.

Restriction enzyme analysis and electrophoresis

The mtDNA was digested with restriction enzyme Sau3A or BglII for 2-3 hours at 37° C using the buffers recommended by the manufacturers (Bethesda Research lab, Inc. Gaithersburg, MD., [BRL]) The fragments were separated by electrophoresis in 0.7% agarose gels using TBE buffer (Maniatis et al, 1982). Gels were electrophoresed at room temperature for 18 hours at 35-40 volts. The gels were stained with ethidium bromide (0.5 µg/ml) for 15-20 min and photographed. The restriction fragments on the gel were transferred to nylon hybridization membranes (MSI. Westboro, MA.) as described by Maniatis et al (1982). The DNA was fixed on the membrane by UV crosslinking for 3-5 min.

Isolation and cloning of the RFLP fragment

Mitochondrial DNA of CL25 was digested with restriction enzyme Sau3A and electrophoresed in agarose gel as described above. After staining, agar containing the 2.5 kb RFLP fragment was cut out from the gel and the DNA was electroeluted with an electroeluter using the procedure described by the manufacturer (Schleicher and Schuell) in TBE buffer. The buffer with the electroeluted DNA was precipitated overnight with 2 volumes of absolute EtOH and 1/10 volume of NaOAc at 0°C. The DNA fragment was collected by spinning at 15K for 50 min.

The 2.5 Kb Sau3A fragment was cloned into the BamH1 site of polylinker of the cloning vector pUC118. The recombinant plasmid pSK51 was isolated using a boiling miniprep method described by Maniatis et al (1982).

DNA labelling and hybridization

To release the cloned mtDNA insert, the recombinant plasmid was digested with restriction enzymes HindIII and EcoR1 and electrophoresed in an agarose gel. Agarose containing the portion of the recombinant plasmid insert was cut out of the gel, electroeluted with an electroeluter using the procedure suggested by the manufacturer (Schleicher and Schuell). The electroeluted DNA was used for probes after radiolabelling. Probes were radiolabelled using the random primer method (Feinberg and Volgenstein, 1983).

Cytoplasmic fusion via strain pairing

Resistance to 8 mg chloramphenicol/ml and a light pigment phenotype were used as mitochondrial and nuclear markers, respectively, for strain CL25. This strain was paired with 4-C, a chloramphenicol-sensitive, virulent isolate with orange pigmentation. Cytoplasmic

fusion was performed by placing inoculum plugs of the two isolates side by side approximately 5mm apart on cellophane layered over PDA. The isolates were allowed to grow and fuse for 5 days. The cellophane and fusing cultures were lifted off the PDA and placed on PDA supplemented with 8 mg chloramphenicol/ml. After 5-7 days, plugs of mycelia from the side of the fused strains containing the orange pigmented, virulent strain were removed and subcultured on PDA supplemented with 8 mg chloramphenicol/ml. Subcultures that were orange and able to grow on PDA with chloramphenicol were grown in liquid media with and without chloramphenicol. The mtDNA from these subcultures was extracted to determine the presence or absence of the RFLP associated with CL25 mtDNA.

RESULTS

RFLP analysis of mtDNA

Mitochondrial DNA of CL25 digested with restriction enzyme Sau3A contained a unique fragment of about 2.5 kb, while all other C. parasitica strains contained two smaller bands not observed in CL25. The presence of this fragment did not seem to correlate with the hypovirulence phenotype in CL25 since a virulent single conidial isolate of strain CL25 also contained this band. A clone of the 2.5 kb fragment of CL25 (pSK51) was used to screen CL25 and other strains. Clone pSK51 hybridized to the 2.5 kb fragment in CL25 and to the two smaller bands in 4-C (Fig 2.1-B, lane 1 and 4) and other strains. This RFLP pattern was subsequently used to detect CL25 and 4-C mtDNA after hyphal fusion.

Isolation of chloramphenicol resistant strain of CL25

Growth of CL25 (and other strains) was greatly reduced at 6 mg chloramphenicol/ml, but a better discrimination between chloramphenicol resistant and sensitive strains could be

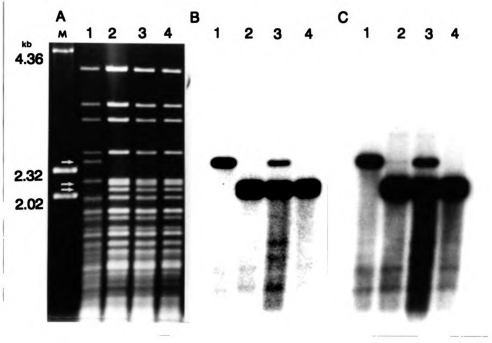

Fig 2.1 - Sau3A digestion pattern of mtDNA of different isolates of C. parasitica. Lane 1, CL25. Lane 2, 4-C(H) grown in broth (without chloramphenicol). Lane 3, 4-C(H) grown in broth supplemented with chloramphenicol. Lane 4, 4-C.
A - Ethidium bromide stained gel, M= λ DNA digested with HindIII used as molecular size marker.
B - Southern blot hybridized with a 2.5 kb fragment clone of CL25 mtDNA. Polymorphic bands in the gel are indicated with arrows.
C - A longer exposure of the blot B.

obtained at 8 mg chloramphenicol/ml or greater concentration. A CL25 strain resistant to 8 mg chloramphenicol/ml was isolated on PDA plates containing 8 mg chloramphenicol/ml. The chloramphenicol resistant isolates (CL25 cap^R) tolerated chloramphenicol at high concentrations when compared to sensitive isolates. The chloramphenicol resistant isolate grew at a normal rate on PDA when compared to the sensitive strains.

To demonstrate that chloramphenicol resistance was maternally inherited and therefore mitochondrial-associated in C. parasitica, reciprocal crosses using CL25 cap^R and 4-C cap^S were made and ascospores were collected from each cross. These ascospores were grown on PDA and PDA supplemented with 8 mg chloramphenicol/ml. Cultures from ascospores collected from 8 perithecia in which the maternal parent was chloramphenicol resistant were able to grow in the presence of chloramphenicol, whereas when CL25 cap^R was the paternal parent, none of the ascospore cultures from 16 perithecia grew on media supplemented with chloramphenicol.

Transfer of mitochondria during hyphal anastomosis

To determine if mtDNA from CL25 cap^R could transfer via anastomosis to 4-C cap^S, the isolates were paired as described. After pairing, subcultures from the 4-C cap^S side of the pairing that grew on media amended with chloramphenicol were selected. Each subculture was divided and grown in broth with and without the addition of chloramphenicol. The mitochondrial DNA of one subculture 4-C(H), selected on chloramphenicol, showed the RFLP of 4-C when it was grown without chloramphenicol (Fig 2.1-B, lane 2). However, when grown in the liquid media supplemented with chloramphenicol, it showed the RFLP pattern of both CL25 and 4-C (Fig 2.1-B, lane 3). Upon longer exposure of the same blot, the mtRFLP of CL25 appeared as a faint band in the mtDNA of 4-C(H) grown in the liquid

TABLE 2.2. Pathogenicity test of virulent, hypovirulent and converted hypovirulent strains of Cryphonectria parasitica.

Strain	Pathogenicity tests[a] (mm^2)	Description
CL1-16	89	Virulent strain without dsRNA.
CL25 cap^R	10	A light pigmented chloramphenicol resistant CL25 strain.
4-C	76	An orange pigmented strain derived from GHU4 by curing of dsRNA (Fulbright, 1985).
4-C(H)	12	Strain 4-C converted with CL25 hypovirulence.

[a]Apple fruit were inoculated by removing a 9 x 7-mm-deep plug of apple tissue and inserting mycelium of strain to be tested. Three weeks later the area of the infected discolored region was calculated (Fulbright, 1984).

Table 2.3. Phenotypic and genotypic characteristics of single-conidial isolates of strain 4-C(H).

SCI	Virulence[a]	RFLP	Cap$^{R\ or\ S}$	Pigmentation
#1	H	4-C	S	Orange
#2	V	4-C	S	Orange
#3	V	4-C	S	Orange
#4	V	4-C	S	Orange
#5	V	4-C	S	Orange
#6	H	4-C	S	Orange
#7	H	4-C	S	Orange
#8	H	CL25	S	Orange
#9	H	4-C	S	Orange
#10	V	4-C	S	Orange
#11	H	4-C	S	Orange
#12	V	CL25	S	Orange
CL1-16	V	NA	S	White
CL25 Cap^R	H	CL25	R	white
4-C(H)	H	4-C and CL25	R	Orange

SCI= Single-conidial isolates, S= Cap^S, R= Cap^R, H= Hypovirulent, V= Virulent, NA= Not applicable.

[a]Apple fruit were inoculated by removing a 9 x 7-mm-deep plug of apple tissue and inserting mycelium of strain to be tested. Three weeks later the area of the infected discolored region was calculated (Fulbright, 1984).

broth without chloramphenicol (Fig 2.1-C, lane 2), indicating either a mixture of cultures or a mixture of mitochondria within the same thallus. To determine if the light-pigmented CL25 hyphae might be contaminating the orange-colored 4-C(H) thallus, single-conidial isolates were obtained from the subculture. All single-conidial isolates recovered were orange indicating that they had the 4-C nuclear type. Twelve of these single-spore isolates were analyzed for their RFLP pattern. Ten single-spore isolates showed the mitochondrial RFLP banding pattern of 4-C and two showed the mitochondrial banding pattern of CL25 (Table-2.3). A virulence assay performed with 4-C(H) demonstrated that this strain was reduced in virulence similar to CL25 cap^R (Table-2.2).

DISCUSSION

Nuclear and mitochondrial markers were required to determine whether or not mitochondria could transfer during vegetative fusion. Pigmentation of C. parasitica has been described as a nuclear trait by Anagnostakis (1982) and therefore, the naturally light pigmentation phenotype of CL25 and the orange pigmentation phenotype of 4-C were used as nuclear markers.

Both genetic and molecular mitochondrial markers were constructed. Chloramphenicol resistance has been shown to be a genetically stable extrachromosomal trait associated with the mitochondria of many other fungi such as Ophiostoma ulmi (Brasier and Kirk, 1986), Coprinus cinereus (Baptista-Ferreira et al,1983), Saccharomyces cerevisiae (Borst and Grivell, 1978; Dujon B., 1980), Aspergillus nidulans (Gunatilleke et al, 1975), and Podospora anserina (Belcour and Begel, 1977). Chloramphenicol resistance in CL25 also was shown to be a cytoplasmic trait in the study described above. These data lend strong support to the concept that cap^R is an allele on the mtDNA. Restriction digest analysis with Sau3A revealed a

mitochondrial RFLP of a 2.5 kb band which was unique to CL25. Although this RFLP serves as a molecular marker for CL25 mitochondrial DNA, it is not likely to have a role in hypovirulence since a virulent single spore isolate of CL25 also contains this fragment. After vegetative pairing between CL25 cap^R and 4-C, a subculture, 4-C(H), was selected that showed the nuclear trait of 4-C and the mitochondrial RFLP of both CL25 and 4-C (Fig 2.1-C, lane 2). These results strongly suggest that mitochondria have moved from CL25 to 4-C via hyphal fusion. The increased intensity of the mitochondrial RFLP unique to CL25 when 4-C(H) was grown continuously in the presence of chloramphenicol is clearly due to the fact that chloramphenicol enhances the selection of the cap^R mitochondrial population within the 4-C(H) thallus.

Virulence tests with 4-C(H) indicated that this heteroplasmic strain became hypovirulent after vegetative fusion. Thus, the mitochondrial genome and the factor responsible for the hypovirulence phenotype are capable of transfer during hyphal fusion. This supports the hypothesis that mitochondria are associated with the dsRNA-free hypovirulence phenotype found in CL25 and perhaps other strains showing the same phenotype.

To eliminate the possibility that CL25 hyphae might be contaminating the pigmented strain, single-conidial isolates of 4-C(H) were selected. All single spore cultures contained the pigment nuclear marker of 4-C. Out of twelve single-spore isolates that were screened for their RFLP pattern, two of those contained the mitochondrial RFLP marker of CL25, thus eliminating the possibility of hyphal contamination. Virulence analysis with the twelve single-conidial isolates of 4-C(H) demonstrated that five of these isolates were hypovirulent and seven of them were virulent. All twelve of these isolates did not grow in the presence of chloramphenicol, and thus are cap^S (Table- 2.3). These data suggest that the strain 4-C(H) is a heteroplasmon and the single conidial isolates are the result of non-reciprocal

recombination between DNAs of the two different types of mitochondria in 4-C(H) and their subsequent vegetative segregation. If all the loci are carried by the mitochondrial genome, then these results are reminiscent of polar recombination between mtDNAs of other fungi.

Genetic studies of Neurospora and yeast have shown that recombination between mtDNA occurs frequently in heteroplasmons. Ultimately a single mitochondrial type predominates, be it the either a parental type or recombinant type. When a recombinant type predominates, it often results from unidirectional gene conversion (Manella and Lambowitz, 1978; Manella and Lambowitz, 1979; Dujon, 1980). Examples of gene conversion or non-reciprocal recombination are encountered in Saccharomyces cerevisiae crosses where the polarity of recombination of mitochondrial alleles is determined by the ω locus on the mitochondrial DNA. ω^+ differs from ω^- by the presence of an intron (1143 bp) in the 21S rRNA gene; polarity of recombination originates at the ω locus (Borst et al, 1977; Jacq et al, 1977, Dujon, 1980), with gene conversion favoring the alleles that are linked to ω^+. Although more studies are needed to show conclusively that the CL25 hypovirulence is truly linked to the other markers on the mitochondrial genome, we believe that our genetic evidence supports this data.

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CHAPTER-3

ASSOCIATION OF ELEVATED MITOCHONDRIAL ALTERNATE OXIDASE ACTIVITY WITH THE HYPOVIRULENCE PHENOTYPE OF CRYPHONECTRIA PARASITICA.

ABSTRACT

Hypovirulent strains of Cryphonectria parasitica, the causal agent of chestnut blight have been associated with the presence of cytoplasmically transmissible double stranded RNA (dsRNA). Recently, several hypovirulent strains have been found that are not associated with detectable levels of dsRNA. Respiration studies of dsRNA-free hypovirulent strains revealed the presence of an induced alternate oxidase pathway and possibly defects in the electron transport system. We present evidence that alternate oxidase can be induced in other C. parasitica strains after they undergo hyphal anastomosis with a dsRNA-free hypovirulent strain. Some of these dsRNA-free hypovirulent strains had a slower growth pattern compared to virulent strains. These results suggest that the hypovirulence phenotype, altered growth pattern and the induction of the alternate oxidase result from a defective cytochrome mediated electron-transport. This work indicates that the phenotype known as hypovirulence may have more than one cause and that the mitochondrial genome may, in some cases, be responsible for hypovirulence in C. parasitica.

INTRODUCTION

The mitochondria of higher plants and fungi have a branched electron transport chain with a cyanide-sensitive cytochrome pathway that terminates with cytochrome oxidase and a cyanide-resistant, hydroxamic acid sensitive alternative pathway that terminates with an alternative oxidase (Lambowitz and Zannoni, 1978). Both oxidases reduce oxygen to water as the end product of electron transport (Solomos T. 1977). When electrons flow through the cytochrome pathway, energy is conserved in the form of ATP, but when the electrons flow through the alternate oxidase pathway, the potential energy of the system is lost as heat. In Sauratum guttatum (voodoo lily), a member of family Araceae, the alternate pathway is most highly expressed in thermogenic tissue, where its function is to generate heat to attract insects, thereby facilitating pollination. This is probably the best understood physiological role of alternate oxidase (Elthon and McIntosh, 1987). In fungi, characterization of the alternative pathway has been described best in the poky mutant of Neurospora, which has a conspicuously slow growth rate. In this mutant the alternate pathway accounts for 70% of the total respiration (Mitchell et al,1952). The mitochondria of poky contain abnormally low amounts of cytochrome aa_3, b and 30% more soluble cytochrome c and flavoproteins (Tissieres et al.1953, Lambowitz et al. 1972; Jagow et al, 1973; Bertrand et al. 1976). Alternate oxidase can be induced in wild-type cells by growing the mycelium in the presence of chloramphenicol, antimycin A (Lambowitz and Slayman, 1971) or oligomycin (Szakacs and Bertrand, 1976; Bertrand et al, 1976), which are inhibitors of mitochondrial protein synthesis, electron transport, and ATP synthesis, respectively.

In this chapter, I investigated the possibility that respiratory deficiencies could be a factor responsible for the reduced virulence (hypovirulence) phenotype of Cryphonectria (Endothia) parasitica (Murr.) Barr., the causal agent of chestnut blight. Chestnut blight was

responsible for the demise of the once prevalent American chestnut (Castanea dentata [Marsh] Borkh) in North America. Isolates with reduced virulence (hypovirulent) have been isolated where chestnut trees survive infection. The well-characterized hypovirulent strains of C. parasitica have been found to be infected with cytoplasmically transmissible, double-stranded RNA (dsRNA) molecules which are correlated with changes in virulence, growth rate, sporulation and/or culture morphology. Several isolates of C. parasitica, including CL25 from Crystal Lake, Michigan, have the same characteristics of dsRNA-associated hypovirulence but harbor no detectable dsRNA. Genetic studies of CL25 show that the hypovirulence phenotype is carried in 20% of the conidia, is maternally inherited and is cytoplasmically transferred through hyphal anastomoses (Fulbright, 1985). These studies suggested that there is a cytoplasmic "virulence inhibition factor" (VIF, Fulbright (1990)) other than dsRNA that might be involved in the hypovirulence phenotype. Because of the cytoplasmic nature of the hypovirulence phenotype, I investigated the possibility of mitochondrial defects in the dsRNA-free strains of C. parasitica. Evidence is presented that supports a positive correlation between the presence of high levels of alternate oxidase and the dsRNA-free type of hypovirulence.

MATERIALS AND METHODS

Strains and Growth Condition

The strains or isolates of C. parasitica used in this study are listed in Table 3-1. Cultures were maintained on potato dextrose agar (PDA, Difco) at 20°C under fluorescent light. Fungal cultures were grown at room temperature for 5 days in the Endothia complete liquid medium (ECM) of Puhalla and Anagnostakis (1971) modified by the omission of glucose. Chloramphenicol (Sigma) was added to the cultures, when needed, from a stock

TABLE 3.1. Alternate oxidase pathway as percentage of total respiration in different strains of Cryphonectria parasitica.

Strains	Description	Phenotype	Presence of dsRNA	Alt. oxd. as % of total respiration
EP155*	From Connecticut	V	-	9.0
CL1-16*	SCI from a standard virulent strain from Frankfort, MI.	V	-	10.5
14B-N1	SCI from a strain from Kellogg forest, MI.	V	-	17.5
4-C*	dsRNA cured GHU4 (Fulbright,1984).	V	-	8
CL25 SS#4*	SCI from a strain from Crystal Lake, MI.	V	-	13.5
CL25 SS#9*	SCI from a strain from Crystal Lake, MI.	HV	-	71.5
14B-D6*	SCI from a strain from Kellogg forest, MI.	HV	-	84.5
14B-N6*	"	HV	-	71.0
4-C(H)*	Strain 4-C converted with CL25 type of hypovirulence, contains the mitochondria of both strains.	HV	-	82.0

TABLE 3.1. (cont.)

Strains	Description	Phenotype	Presence of dsRNA	Alt. oxd. as % of total respiration
GHU4*	From Grand Haven, MI.	HV	+	53.0
GH2*	"	HV	+	16.5
CL1-16(GH2)	Strain CL1-16 converted with GH2 type of hypovirulence.	HV	+	15.0
R1	From Roscommon, MI.	HV	+	6.5

V = previously determined to be virulent (unpublished)
HV = previously determined to be hypovirulent (unpublished)
SCI= Single conidial isolate.
Strains with * represent averages of multiple samples.

solution in 95% ethanol to a final concentration of 2 mg/ml. The final concentration of ethanol was always less than 1% (v/v) and this concentration did not affect respiration in C. parasitica.

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.) connected to a Gould recorder. The respiration measurements were made using several modifications of the basic techniques previously described for Neurospora by Lambowitz and Slayman (1971). Four-to-five day-old-cultures grown in ECM were homogenized for 15-20 seconds using a Polytron homogenizer. The reaction was begun by pipetting 3 ml of the homogenized mycelium into a reaction chamber. The sample was aerated for about 1 min to a final oxygen concentration of about 240 ppm. The electrode was quickly inserted into the reaction chamber and the oxygen consumption recorded. The reaction chamber was maintained at 25°C and the mycelial suspension was constantly mixed with a magnetic stirring bar.

Potassium cyanide (KCN; Aldrich) and salicylhydroxamic acid (SHAM; AnalaR), 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 added separately to the reaction chamber during measurements of oxygen consumption. SHAM was added from a 50 mg/ml solution in 95% ethanol to a final concentration of 2.7 mM, and KCN was added to a final concentration of 0.8 mM from a 0.1 M solution in 10 mM Tris-HCl, 5 mM EDTA, pH 7.2. Both inhibitors were freshly prepared prior to each experiment.

Isolation of mitochondria

For the determination of enzyme activities, mitochondria were isolated by a modification of the procedure of Bertrand and Pittenger (1969). Five-to-seven day-old-cultures grown in ECM were harvested by suction filtration. The mycelial mat was rinsed once with deionized water, followed by a wash with sucrose-EDTA isolation buffer (0.44 M sucrose, 5 mM EDTA, 10 mM Tris-HCl; pH 7.4). All the following steps were performed at 4°C. Cells were disrupted by grinding the mycelial mat with acid-washed sea sand in a cold mortar and pestle. The mycelial paste was suspended in 20 to 30 ml of isolation buffer. This was followed by two successive 10 min centrifugations at 2500Xg (4,500 rpm, Sorvall 55-34 rotor) to pellet all debris, nuclei and sand. Mitochondria were pelleted by centrifugation at 25,000Xg for 45 min (14,500 rpm, Sorvall SS-34 rotor).

Determination of enzyme activities

Mitochondria isolated as described above were used to determine enzyme activities. In all cases, mitochondrial pellets were resuspended in a buffer containing 5 mM EDTA., 10 mM Tris-HCl; pH 7.2.

Cytochrome oxidase activity

To determine cytochrome oxidase activity, the oxidation of reduced cytochrome c was followed spectrophotometrically at 550 nm, as described by Smith (1955) and modified by Nargang (1978). Reduced cytochrome c was obtained by adding a few crystals of sodium dithionite to a 10 mg/ml solution of cytochrome c. Excess dithionite was removed by bubbling air through the mixture for 1 min 50 µl of the reduced cytochrome c solution was added to 900 µl of TE buffer (5 mM EDTA., 10 mM Tris-HCl; pH 7.6) and the reaction was started

by addition of 10 µl of the mitochondrial suspension. This was mixed quickly and the absorbance was determined as above.

Succinate oxidase activity

Succinate oxidase was determined by a modification of the procedure described by Stotz (1955). One ml of the reaction mixture contained 50 µl of 10 mg oxidized cytochrome c/ml, 880 µl of TE buffer (5 mM EDTA., 10 mM Tris-HCl; pH 7.6), 50 µl of 35 mg succinate/ml, 10 µl of 0.1M KCN and 10 µl of the mitochondrial suspension. The activity was determined by spectrophotometrically following the reduction of cytochrome c at 550 nm.

NADH oxidase activity

NADH oxidase activity was determined in the same manner as described for succinate oxidase with NADH substituted for the succinate.

Determination of dry weight

Cryphonectria parasitica strains were grown in Endothia minimal media (Puhalla and Anagnostakis (1971). After 10 days of growth, the liquid medium was poured off the fungus and the mycelial mass was air dried. When the fungal strains dried completely, their weight was determined by scraping the mycelial fragments into preweighed weightboats.

RESULTS

Respiration

To determine if C. parasitica isolates with the hypovirulence phenotype were affected by mitochondrial defects, respiration was measured and compared between hypovirulent and

virulent strains. The isolates studied could be placed into one of four different respiration patterns (Fig 3-1, A-D). In all cases, the initial slope of the curve in figure 3-1 represents 100% respiration. When an inhibitor of the cytochrome pathway, cyanide, is added, the electron flow is directed towards the alternate pathway. Subsequently, when SHAM, the alternate oxidase inhibitor, is added, respiration through the alternate pathway is inhibited, and there is very little residual respiration. As seen in the altered rate of oxygen utilization in the reactions, all virulent strains and most dsRNA-associated hypovirulent strains were inhibited by the addition of cyanide (Fig 3-1, A and C), with the exception of GHU4 (Fig 3-1, D). In contrast, respiration was only moderately inhibited by the addition of cyanide in the dsRNA-free hypovirulent strains (Fig 3-1, B), since the curves representing the rate of oxygen utilization did not change to a large extent as compared to the virulent and dsRNA-associated type of hypovirulent strains (Fig 3-1, A and C). The alternate oxidase pathway represented a significantly high percentage of the total respiration in these isolates (Table 3-1). These results indicate that there is an association of increased alternate oxidase activity with the hypovirulent phenotype, and a correlation of this phenotype with the dsRNA-free hypovirulence state. To determine if the alternative oxidase pathway was inducible in strains showing low levels of alternative oxidase, some of these isolates were grown in the presence of chloramphenicol. The alternate oxidase was induced up to about 8 fold over the normal amount (Table 3.2).

When virulent strain 4-C was paired with hypovirulent strain CL25, the converted strain 4-C(H) had respiration characteristics that resembled CL25 with the alternate oxidase pathway accounting for 82% of the total respiration. Before conversion only 8% of the total respiration of 4-C occurred through the alternate oxidase pathway (Table-3.1). The increase

Fig 3.1- Oxygen electrode recordings of the respiration of different strains of Cryphonectria parasitica. (A) EP155 [virulent] (B) CL25 #9 [hypovirulent without dsRNA] (C) GH2 [hypovirulent with dsRNA] (D) GHU4 [hypovirulent with dsRNA] (E) Control- Respiration of strain CL25 without any addition of respiratory inhibitors. KCN= Potassium cyanide, SHAM= Salicylhydroxamic acid.

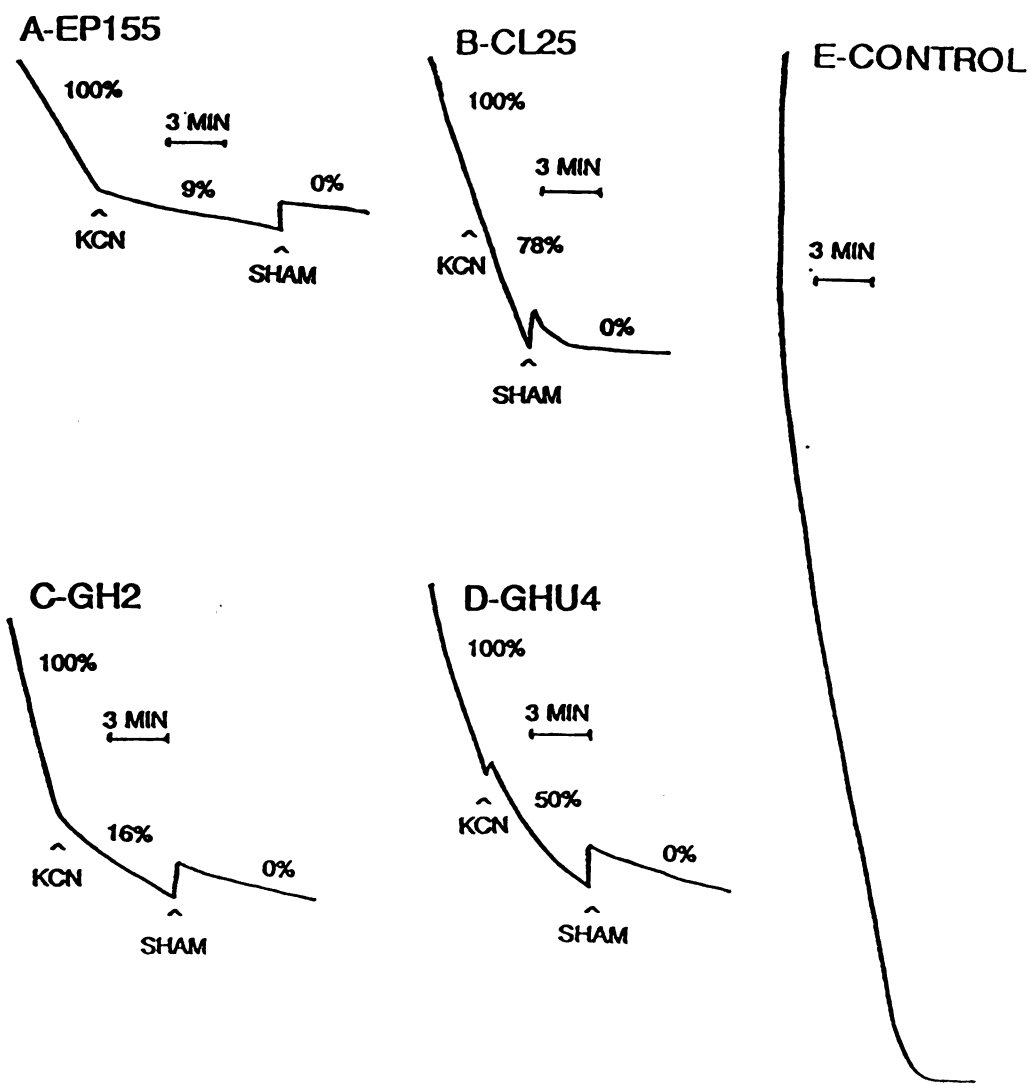

Table 3.2- Induction of alternate oxidase pathway with chloramphenicol in different strains of Cryphonectria parasitica.

	Alt. ox. as % of total respiration	
Strains	***+chloramphenicol**	**-chloramphenicol**
CL1-16	10.5	80
GH2	16.5	42
CL1-16(GH2)	15	62

* Cultures were grown in Endothia complete media amended with 2 mg/ml chloramphenicol for 5 days.

in respiration through the alternate pathway in strain 4-C(H) implies that the induction of alternative pathway may be a manifestation of mitochondrial disfunction and that this disfunction is transferred to other strains through hyphal anastomosis.

Enzyme assays

The enzymatic activities of cytochrome c oxidase, succinate oxidase and NADH oxidase were determined in isogenic strains of CL25 (Table 3.3). The ratio of cytochrome c oxidase to NADH dehydrogenase was found to be the same in both virulent and hypovirulent strains. The ratio of cytochrome c oxidase to succinate oxidase was two-fold higher in the virulent isogenic single-conidial isolate CL25#4 than in the dsRNA-free hypovirulent isogenic single-conidial isolate CL25#9. These results suggest that the hypovirulent strain CL25#9 may be deficient in cytochrome oxidase activity.

Dry weight of different strains

Determination of dry weight of different strains after growth in broth for 10 days showed that the converted hypovirulent strain 4-C(H) had a dry mycelial mass of about 43 mg as compared to about 133 mg in virulent strain 4-C (Table 3.4). Hypovirulent single-conidial isolate, CL25#9, had a dry weight of 80 mg and the dry weight of a virulent control strain CL1-16 was similar to virulent strain 4-C (Table 3.4).

DISCUSSION

Clonal deterioration and cellular death occurs in filamentous fungi after prolonged asexual propagation. This phenomenon is described as senescence, and examples of some fungi that experience vegetative senescence are Podospora, Neurospora and Aspergillus.

Table 3.3- The activities of cytochrome c oxidase, succinate oxidase and NADH oxidase in partially purified mitochondrial preparations of isogenic strains of CL25.

Strains	CCO min-1	SO OD/min	NADH oxd. OD/min	CCO/ NADH oxd.	CCO/SO
CL25#9	0.154	0.092	0.216	0.428	1.67
CL25#4	0.088	0.024	0.059	0.404	3.6

Abbreviations-

CCO= cytochrome c oxidase
SO= succinate oxidase
NADH oxd= NADH oxidase

Table 3.4- Determination of dry weight of different strains of C. parasitica grown in Endothia minimal broth.

	Dry weight* of different strains in mg.			
Replicates	**4-C**	**4-C(H)**	**CL25#9**	**CL1-16**
1	120	40	80	150
2	130	40	80	160
3	150	50	80	150
Mean	133.3	43.3	80	153.3

*Dry weight of different C. parasitica strains were determined by growing two 9mm plugs of mycelium (cut with a cork borer) in Endothia minimal media. After 10 days, the broth was poured off the mycelial mass, air dried completely and weighed.

Some of the common symptoms of senescence are female sterility, respiratory abnormalities, and cytochrome aa_3 and b deficiencies in the mitochondria (Bertrand, 1983). There is conclusive evidence that senescence in fungi is caused by mutations affecting the mitochondrial genome.

"Diseased" isolates of Ophiostoma ulmi, the pathogen of Dutch elm disease, display a slow growth rate, reduced viability of conidia and impairment of sexual reproduction. The diseased state is transmitted by extrachromosomal elements, referred to as d-factor, and is correlated with the transmission of dsRNA (Brasier, 1983). A recent study suggests that the diseased state is related to a deficiency in cytochrome aa_3 in dsRNA infected isolates. The dsRNA in these strains copurifies with the mitochondria. It has been postulated that the debilitation of growth may be due to suppression of respiration in diseased strains (Rogers et al. 1987).

Our results indicate that C. parasitica utilizes two oxidase systems, one sensitive to SHAM and the other sensitive to cyanide. We showed that alternate oxidase accounted for a high percentage (up to 85%) of the total respiration in dsRNA-free hypovirulent strains as opposed to 6-20 % of total respiration in the virulent and dsRNA-associated types. The presence of higher percentage of alternate oxidase in dsRNA-free hypovirulent strains is reminiscent of that found in senescing strains of Neurospora and other fungi, in which there is a branched electron transport system. The correlation of dsRNA-free hypovirulence with a reduced growth rate is consistent with the altered rate of alternate oxidase and cytochrome oxidase activities. To determine if respiratory defects are affecting the growth of C. parasitica, some of the strains were grown in Endothia minimal media, where the growth of the fungus is restricted by the concentration of the materials. Therefore, the fungus depends on its own metabolism for energy and if there is any defect in the respiration, it should become apparent.

Dry weight measurements of different strains showed that the growth of converted strain 4-C(H) is much slower than that of virulent strain 4-C. Also the hypovirulent single-spore isolate of CL25 had a slower growth rate than either of the two virulent strains tested. This study suggests that the induction of the alternate oxidase pathway may have an effect on the growth of the fungus. Furthermore, assays with isogenic strains of CL25 differing in virulence and the amounts of alternate oxidase show that the hypovirulent strain CL25#9 may be deficient in cytochrome oxidase activity. Thus it appears that hypovirulence, altered growth phenotype and the induction of the alternate oxidase result from a defective cytochrome-mediated electron transport.

In Podospora, Aspergillus, and Neurospora, the extranuclear factors responsible for respiratory defects and subsequent senescence of the culture spread to normal cultures after hyphal fusion between senescent and normal hyphae (Bertrand, 1983). This conversion of normal hyphae to a senescent state reveals that these factors are invasive or "suppressive" in nature. A similar process may function in C. parasitica, since hypovirulence is determined by cytoplasmically transmissible factors that can convert virulent strains to hypovirulent via hyphal anastomosis. Respiration studies of a converted hypovirulent strain 4-C(H) revealed that the alternate oxidase pathway accounted for 82% of the total respiration compared to 8% before conversion. The strain 4-C(H), was found to contain a mixture of mitochondria from both 4-C and CL25 (chapter 2). The original CL25 is also probably a heteroplasmon since only 20 % of the single-conidial isolates of CL25 inherit hypovirulence (Fulbright, 1985). From these data, we assume that only a minority of the total mitochondria are defective. Our speculation at this point is that these defective mitochondria are "suppressive" in nature and if they exceed a certain proportion of the population (about 20%), it is lethal to the fungus. Our observation regarding mitochondrial DNA recombination in the single-conidial analysis

of the heteroplasmon 4-C(H) (chapter 2) supports this theory since many rounds of non-reciprocal polar recombination could account for the suppressivity of the hypovirulent mitochondrial genome. These observations suggest that the induction of alternate oxidase may be associated with the hypovirulence phenotype in dsRNA-free strains.

Since altered growth morphology and debilitation are common factors associated with hypovirulence in C. parasitica, the "diseased" state of O. ulmi, and senescence in Neurospora and other fungi, it is important to also note that these phenotypes may share a common mitochondrial disfunction. Thus studies on one system may help us understand the other. This is a new direction to explore in connection with the phenomenon of hypovirulence.

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CONCLUSION

Hypovirulent strains of Cryphonectria parasitica have been correlated with the presence of double-stranded RNA (dsRNA). Recently, several hypovirulent strains have been discovered that do not have detectable amounts of dsRNA. Due to the cytoplasmic nature of hypovirulence, I decided to study the role of extrachromosomal factors that might be involved in dsRNA-free hypovirulence. The presence of a 6 kb plasmid in the mitochondria of all virulent and hypovirulent strains of C. parasitica assayed reduced the possibility that this plasmid might have a role in the dsRNA-free hypovirulence phenotype. However, this finding does not eliminate the possibility of other mitochondrial-borne plasmids or other plasmids causing hypovirulence.

Due to the low level of virulence and the maternally inherited nature of dsRNA-free hypovirulent strains, a mutation in the mitochondrial genome was suspected. Mitochondrial mutations may involve respiratory defects, such as those which are responsible for the debilitation and senescence of Neurospora, Podospora and Aspergillus and possibly Ophiostoma ulmi. Respiration studies of the dsRNA-free hypovirulent strains revealed that cyanide-insensitive respiration (the alternate oxidase pathway) accounted for as much as 85% of the total respiration. Virulent strains and dsRNA-associated hypovirulent strains (except one) had a very low percentage of total respiration occurring through alternate oxidase pathway (up to 16%) when compared to the dsRNA-free hypovirulent strains. A virulent strain having only 8% of its total respiration through the alternate oxidase pathway was converted to the hypovirulence phenotype by a dsRNA-free hypovirulent strain. After conversion, the alternate oxidase pathway accounted for 82% of the total respiration indicating that the factor responsible for inducing hypovirulence and cyanide insensitive respiration co-transferred during hyphal fusion. Determination of dry weight of this strain

before and after conversion showed that the growth rate of the converted strain was much slower in minimal media after conversion. We believe that the growth of the fungus is restricted due to a mitochondrial defect and this growth debilitation makes the fungus less capable of infecting its host plants.

The converted strain has been shown to be a heteroplasmon with a mixture of mitochondria from both the virulent and the hypovirulent strain used in the hyphal fusion process. Even though the mitochondria of the hypovirulent strain appear to be a minority of the total mitochondrial population within the converted strain, the defect in respiration and the hypovirulence trait appear to be dominant. This may be fully or partially explained through the phenomenon of "suppressivity" observed in other senescing fungi such as Neurospora, Podospora and Aspergillus. In suppressivity, defective mitochondria replace or outcompete normal mitochondria and spread throughout the hyphae. In these well studied fungal species, senescence is associated with the rearrangement of the mitochondrial genome, with suppressivity resulting in the gradual replacement of the wild-type mitochondrial genome by the defective genome. It has also been proposed that the suppressivity may be due to unidirectional gene conversion, and examples of gene conversion can be found in Neurospora and yeast.

In C. parasitica, the mitochondrial defect and the associated hypovirulence may spread by a similar process of suppressivity, but they appear to not totally replace the resident mitochondria, because CL25 and all its derivatives are heteroplasmons. Conceivably, the mitochondrial defect is lethal if homoplasmic. My preliminary data with single-conidial isolates from the converted heteroplasmon showed that chloramphenicol resistance appears to be lost during mitochondrial mixing. The unequal gene frequencies for cap^R/cap^S, hypovirulence/virulence, and the mtRFLPs point to a polar unidirectional gene conversion.

The hypovirulence phenomenon has previously been described as a trait that can be transferred to other strains via hyphal fusion. I have demonstrated a strong correlation between the presence of a high percentage of alternate oxidase and the dsRNA-free hypovirulence phenotype. We also have demonstrated that this trait could be transferred to other strains via hyphal fusion. These results, when taken with the finding of a deficiency in the cytochrome c oxidase activity in a hypovirulent strain makes the dsRNA-free hypovirulence an interesting phenotype requiring further exploration to implicate alternate oxidase in the occurrence of hypovirulence.

In the future, some of these results need to be confirmed using more samples of virulent and hypovirulent strains. I have been able to see different patterns of respiration using several virulent and hypovirulent strains (Table 1). The enzyme activities of cytochrome oxidase, succinate oxidase and NADH oxidase should be determined in all strains listed in Table 3.1 to see if the deficiency of cytochrome c oxidase is specific to hypovirulent strains. The next obvious step is to perform cytochrome spectra of virulent and hypovirulent strains. These experiments will enable us to determine the nature of the deficiency in the cytochrome system that might be causing the respiratory defect.

Another direction will be to determine if the induction of alternate oxidase is correlated with the presence of a higher percentage of cyanide-insensitive respiration out of the total respiration. Immunoblotting experiments using monoclonal antibodies developed against alternate oxidase of Sauromatum guttatum, which seems to be conserved in a variety of different organisms, may help us determine if there is induction of alternate oxidase in respiratory deficient strains.

If the correlation holds well between the presence of alternate oxidase and the hypovirulence phenotype, it will be worthwhile to try to induce alternate oxidase deficient mutations to examine their effect on the hypovirulence phenotype.