Lack of Association of Mitochondrial Plasmids and Pathogenicity in *Nectria haematococca* (*Fusarium solani* f. sp. *cucurbitae*)

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Two linear plasmids, pFSC1 and pFSC2, are present in mitochondria of a highly pathogenic strain (FS37) of mating population I of *Nectria haematococca* (*Fusarium solani* f. sp. *cucurbitae*). Strains derived by curing FS37 of plasmid DNAs were less pathogenic than FS37, suggesting that plasmid DNA played a role in pathogenicity. To test whether the reduced pathogenicity was due to loss of the plasmids, cured strains were recurrently backcrossed to the plasmid-containing parental strain to introduce cured mitochondria into a near wild-type nuclear background.

Pathogenicity tests with backcrossed progeny revealed nuclear inheritance, but not extrachromosomal inheritance, of pathogenicity determining factors. Also, transfer of plasmid-containing mitochondria into a less pathogenic strain did not result in increased pathogenicity. Thus, the original cured strains apparently had sustained nuclear DNA mutations, induced by the curing process, that reduced pathogenicity. Mitochondrial plasmids did not appear to be directly involved in pathogenicity.

Double-stranded DNA plasmids are found in a number of plants and fungi (Garber *et al.* 1986; Pring and Lonsdale 1985). However, the origin and biological role of these plasmids is largely unknown. The presence of plasmids in plant mitochondria is correlated with cytoplasmic male sterility in a number of species (Dixon and Leaver 1982; Levings *et al.* 1980; Palmer *et al.* 1983), but a clear, causal relationship has not been demonstrated. In fungi, plasmids are found in mitochondria, nuclei, and the cytosol, but the majority are associated with mitochondria (Garber *et al.* 1986). The "true" mitochondrial plasmids have no sequence similarity with mitochondrial DNA and have no known function. In contrast, plasmids derived from mitochondrial DNAs, called defective mitochondrial DNAs, are generally associated with mitochondrial dysfunction and vegetative senescence (Grossman and Hudspeth 1985).

A linear plasmid is correlated with reduced pathogenicity and slow growth in *Rhizoctonia solani* (Hashiba *et al.* 1984), and plasmids are correlated with host range in *forma speciales* of *Fusarium oxysporum* pathogenic on crucifers (Kistler and Leong 1986; Kistler *et al.* 1987), but whether the plasmids are responsible for these characteristics has not been tested. The only fungus with a clear plasmid-encoded phenotype is the killer strain of *Kluyveromyces lactis* (Stark *et al.* 1986).

We identified and characterized two linear mitochondrial plasmids in strain FS37 of mating population I of *Nectria haematococca* Berk. & Br. (anamorph: *Fusarium solani* Appel & Wollenw. f. sp. *cucurbitae* Snyd. & Hans.) (Samac and Leong 1988). This fungus causes a stem and fruit rot of plants in the family Cucurbitaceae (Tousson and Snyder 1961). The plasmids, pFSC1 (9.2 kbp) and pFSC2 (8.3 kbp), share no sequence similarity with mitochondrial DNA and

are not associated with vegetative senescence, indicating that they are true mitochondrial plasmids. The plasmid-containing strain was cured of plasmid DNAs by germinating conidia on an ethidium bromide-containing medium. The cured derivative strains were significantly less pathogenic than the plasmid-containing strain on *Cucurbita maxima* 'Pink Banana Squash,' suggesting that the plasmids had a role in pathogenicity (Samac and Leong 1988). However, the reduced pathogenicity could have resulted from mutations induced by the curing process.

The objective of this study was to determine if mitochondrial plasmids play a role in pathogenicity, or if the curing techniques we employed resulted in nuclear mutations that adversely affected pathogenic ability.

MATERIALS AND METHODS

Fungal strains. Strains FS3 and FS37 of *N. haematococca* were obtained from S. N. Smith. Strain FS37 contained plasmids pFSC1 and pFSC2. The curing protocol used to derive strains FS37A, FS37B, and FS37C from FS37, and characteristics of these cured derivatives have been described previously (Samac and Leong 1988). Cured strain FS37A lacked both plasmids, FS37B contained only pFSC2, and FS37C contained only pFSC1. Strain FS3 contained no plasmid DNA and was less pathogenic than FS37 at low inoculum density (Samac and Leong 1988).

Strains FS3 and FS37 are hermaphrodytic and have opposite alleles at the mating type locus (*MAT1*). Mating population I strains of *N. haematococca* are heterothallic and opposite alleles at the mating type locus are required for a fertile cross.

Construction of backcrossed strains. To evaluate whether the curing process induced mutations that decreased pathogenicity, we wanted to compare pathogenicity of FS37 to strains with cured mitochondria and near wild-type FS37 nuclear DNA. These strains were constructed by mating strains FS37A, FS37B, or FS37C as female parents with

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FS3 and then backcrossing progeny with FS37. Six to nine randomly selected ascospores from each F_1 population were germinated on 1.5% water agar and transferred to V-8 agar slants. Each of these progeny was backcrossed as females to FS37 to produce the first backcrossed generation (BC_1). Highly fertile crosses from each mating were selected, and eight to ten ascospore-derived cultures of each backcrossed generation were grown on V-8 agar. Backcrossing to FS37 was continued in this manner to the BC_4 generation.

Crosses were performed with cultures grown for 10 days on V-8 agar by flooding the mycelium (female parent) with a suspension of conidia (male parent). Perithecia with mature ascospores developed 10–14 days after fertilization. All cultures were grown at 20° C in continuous light.

Mitochondria are maternally inherited (Snyder et al. 1975); therefore, use of the cured derivatives and resultant ascospore progeny as female parents ensured that the progeny had mitochondria free of plasmid DNA. Backcrossing should also reduce the number of mutations in nuclear DNA.

The reciprocal crosses, generating strains with FS37 mitochondria and nuclear DNA from cured derivatives, were also done.

To test whether pathogenicity of a strain could be increased by the presence of plasmid-containing mitochondria, we constructed strains with FS37 mitochondria and nuclear DNA from a strain with comparatively low pathogenicity (FS3). The F_1 was generated by mating FS37 as the female parent with FS3. Eight ascospore-derived cultures from the F_1 generation were then crossed as females to FS3 to produce the BC_1 generation. Seven ascospore-derived cultures were grown from each of three strains exhibiting high, medium, and low relative pathogenicity. These cultures were backcrossed as females to FS3. Backcrossing to FS3 was continued in this manner to the BC_4 generation. For comparison, the reciprocal cross of introducing FS3 mitochondria into a FS37 nuclear background was also done.

Pathogenicity tests. Pathogenicity tests were performed by inoculating seeds of C. maxima 'Pink Banana Squash' with approximately 100 conidia per seed as described previously (Samac and Leong 1988). Seed was purchased from L. L. Olds Seed Company, Madison, WI. Results were analyzed by using MINITAB analysis of variance. Pathogenicity tests were carried out by using 10 seeds for each ascospore-derived culture. Thus, in the crosses with

cured derivative strains, the pathogenicity of each backcrossed generation was tested on 80–100 plants. In crosses introducing plasmid-containing mitochondria into FS3, the pathogenicity of each backcrossed generation was tested on 210 plants.

Growth rate determinations. The linear growth rates of FS37, cured derivative strains, and ascospore-derived cultures with cured mitochondria from the BC_4 generation were measured on slants of Czapek-Dox agar as described previously (Samac and Leong 1988).

RESULTS AND DISCUSSION

The differences measured in pathogenicity between the original cured derivatives (FS37A, FS37B, FS37C) and FS37 were significantly different (Fig. 1), confirming results obtained previously (Samac and Leong 1988). However, pathogenicity of the ascospore-derived cultures from the BC_1 through BC_4 generations containing cured mitochondria was not significantly different from pathogenicity of FS37 (Fig. 1). Cultures from the BC_4 generation retained similar plasmid profiles as the original cured derivatives. Total DNA was extracted as described previously (Samac and Leong 1988) and electrophoresed on agarose gels. The plasmid pFSC2 was observed in cultures with FS37B as the original female parent and pFSC1 was observed in cultures with FS37C as the original female parent (data not shown).

Surprisingly, the pathogenicity of strains with plasmid-containing mitochondria and nuclear DNA from cured derivatives (Fig. 2) was not significantly different from the pathogenicity of FS37. Spores with nuclear DNA containing putative mutations may have been selected against or the mutations were replaced with nonmutated DNA during backcrossing.

As seen in previous experiments (Samac and Leong 1988), the difference in pathogenicity between FS3 and FS37 was significantly different (Fig. 3). However, the pathogenicity of cultures from the BC_1 through BC_4 generations with plasmid-containing mitochondria and FS3 nuclear DNA was not significantly different from FS3 (Fig. 3A). Although the female parents chosen for each generation had high, medium, or low relative pathogenicity, the pathogenicity of these cultures was lower in each succeeding backcrossed generation. The pathogenicity of cultures in the BC_1, BC_3, and BC_4 generations from the reciprocal cross, which introduced mitochondria without plasmids from FS3 into a
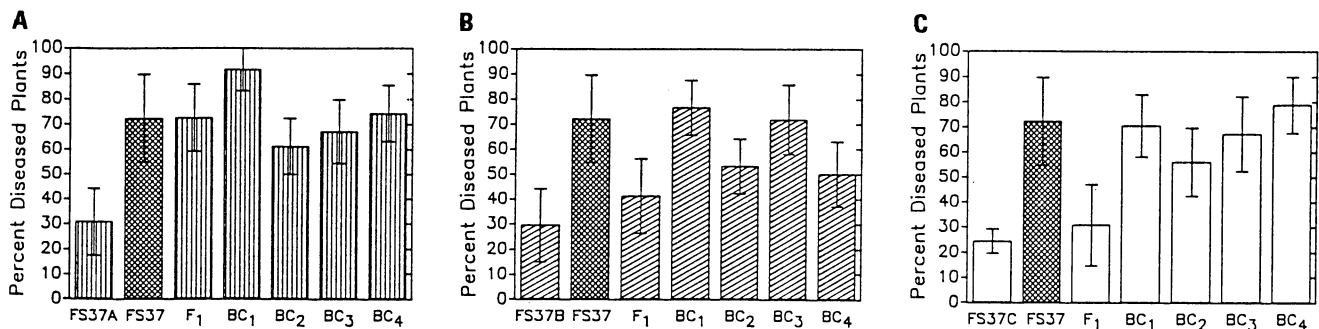

Fig. 1. Relative pathogenicity of parental strains and backcrossed strains with mitochondria from cured derivatives and nuclear DNA from FS37. Bars indicate the mean percentage of diseased plants. Error bars represent 95% confidence intervals. **A**, Strains with FS37A mitochondria lacking both plasmids. **B**, Strains with FS37B mitochondria lacking pFSC1. **C**, Strains with FS37C mitochondria lacking pFSC2.
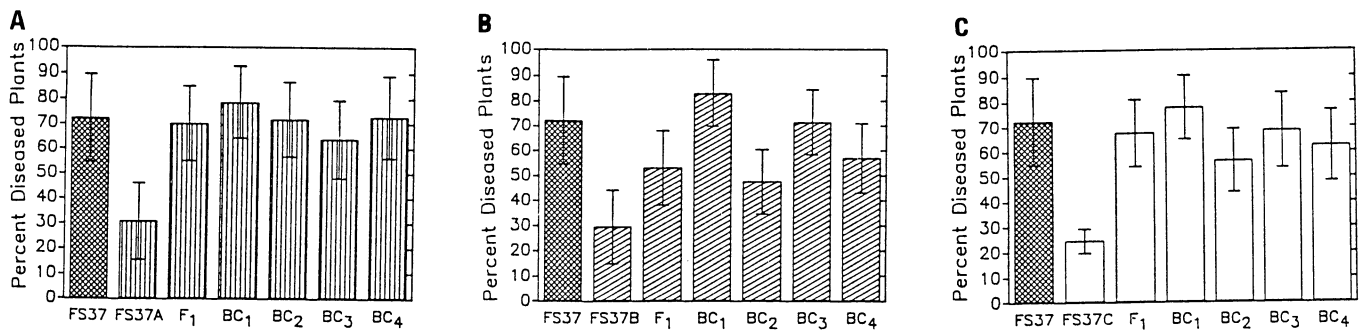
Fig. 2. Relative pathogenicity of parental strains and backcrossed strains with mitochondria from FS37 and nuclear DNA from cured derivatives of FS37. Bars indicate the mean percentage of diseased plants. Error bars represent 95% confidence intervals. **A**, Strains with FS37A nuclear DNA. **B**, Strains with FS37B nuclear DNA. **C**, Strains with FS37C nuclear DNA.
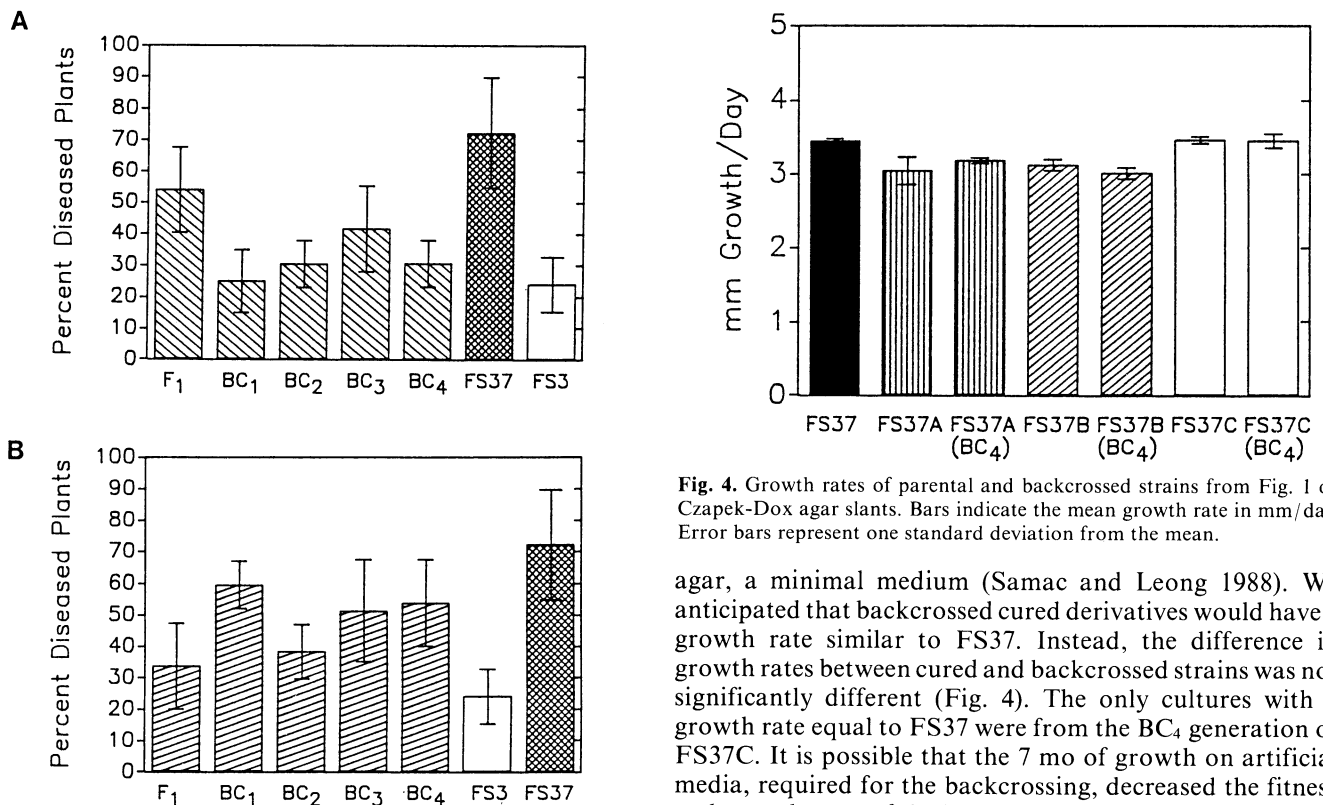

Fig. 3. Relative pathogenicity of backcrossed strains with plasmid-containing mitochondria in an FS3 nuclear background and plasmidless mitochondria in an FS37 nuclear background. Bars indicate the mean percentage of diseased plants. Error bars represent 95% confidence intervals. **A**, Strains with plasmid-containing mitochondria in an FS3 nuclear background. **B**, Strains with plasmidless mitochondria from strain FS3 in an FS37 nuclear background.

Fig. 4. Growth rates of parental and backcrossed strains from Fig. 1 on Czapek-Dox agar slants. Bars indicate the mean growth rate in mm/day. Error bars represent one standard deviation from the mean.

FS37 nuclear background, was not significantly different from FS37 (Fig. 3B). Again, the female parents chosen for each cross had high, medium, or low relative pathogenicity. The pathogenicity of these cultures generally increased in each succeeding backcrossed generation. Thus, combining plasmid-containing mitochondria and FS3 nuclear DNA did not generate a strain with greater pathogenicity than FS3, nor did the absence of plasmids from strain FS37 significantly decrease pathogenicity of FS37 on squash plants.

We observed previously that cured derivative strains grew slower than the plasmid-containing strain on Czapek-Dox
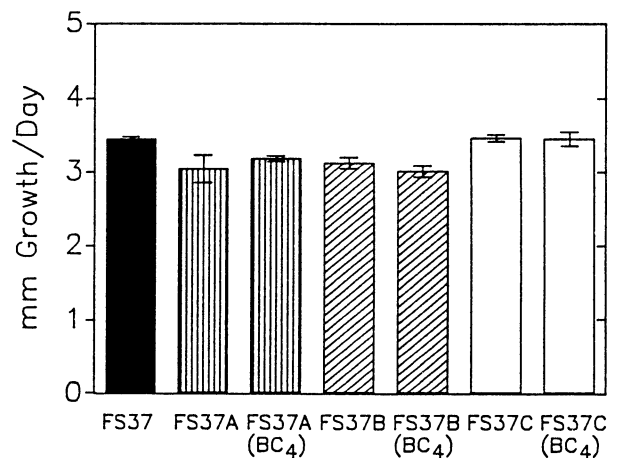
agar, a minimal medium (Samac and Leong 1988). We anticipated that backcrossed cured derivatives would have a growth rate similar to FS37. Instead, the difference in growth rates between cured and backcrossed strains was not significantly different (Fig. 4). The only cultures with a growth rate equal to FS37 were from the BC_4 generation of FS37C. It is possible that the 7 mo of growth on artificial media, required for the backcrossing, decreased the fitness and growth rates of the backcrossed strains compared with FS37.

The results of these experiments indicate that the absence of mitochondrial plasmids from strain FS37 does not modify the pathogenicity of the fungus under test conditions. Additionally, introduction of plasmid-containing mitochondria into a strain with low pathogenicity does not significantly increase pathogenicity of this strain. Thus, the genes that influence the degree of pathogenicity are encoded on nuclear DNA and not extra-chromosomal DNA. The decreased pathogenicity of the original cured derivatives was most likely due to nuclear DNA mutations induced by the ethidium bromide curing process.

Although plasmid DNAs in plants and fungi are frequently present in high copy number, most confer no overt phenotype on the host cell. Curing is one means of establishing whether plasmids play a role in the biology of the organism. Plasmid curing has been successful with only a few fungal plasmids (Koll *et al.* 1984; Niwa *et al.* 1981;

Samac and Leong 1988) and, as this work showed, cured derivatives may contain additional mutations that can alter characteristics of the fungus. The plasmids pFSC1 and pFSC2 do not appear to confer any advantage or disadvantage to strain FS37 and, therefore, may be examples of "selfish" DNA. Selfish DNA makes no contribution to the host phenotype and is devoted only to self replication and survival. For example, the 2μ circle of *Saccharomyces cerevisiae*, a nuclear plasmid, encodes only genes involved in plasmid maintenance (reviewed by Futcher 1988). To establish if mitochondrial plasmids confer a phenotype on their host, molecular techniques, such as transcript mapping and sequence analysis, may be more informative than curing experiments. Such analyses may also shed light on the origins of mitochondrial plasmids.

ACKNOWLEDGMENTS

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