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Linear Plasmidlike DNA in the Plant Pathogenic Fungus *Fusarium oxysporum* f. sp. *conglutinans*

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Double-stranded, 1.9-kilobase-pair (kbp) DNA molecules were found in 18 strains representing three pathogenic races of *Fusarium oxysporum* f. sp. *conglutinans*. The DNA element (pFOXC1) from a race 1 strain and the DNA element (pFOXC2) from a race 2 strain were shown by restriction endonuclease mapping to be linear. pFOXC2 was found in mitochondrial preparations and appears to have blocked 5′ termini, as it was sensitive to 3′ → 5′ exonuclease III but insensitive to 5′ → 3′ λ exonuclease. The major 1.8-kbp *Bgl*II restriction endonuclease fragment of pFOXC2 was cloned in plasmid pUC12. The recombinant plasmid (pCK1) was not homologous to the mitochondrial or nuclear genomes from *F. oxysporum* f. sp. *conglutinans*. This suggests that pFOXC2 is self-replicating. pCK1 was homologous to all 1.9-kbp DNA elements of race 2 but was not homologous to those of race 1 or race 5. All race 1 and 5 elements were also shown to share common DNA sequences.

Linear extrachromosomal DNAs have been observed in numerous fungi (26) as well as in plants (16) and bacteria (2, 3, 7, 30). In cases in which they have been sufficiently described, these elements share common features, including terminal inverted repeats and polypeptides covalently attached to the 5′ end of the molecule, properties similar to those of mammalian adenoviruses (19). These characteristics are important for the 3′-hydroxyl priming of DNA polymerase and subsequent strand displacement during DNA replication (19). Many linear plasmids found in fungi and plants are maternally inherited and appear to be localized in the mitochondria.

We report here the identification of linear plasmidlike DNA in three races of the plant pathogenic fungus *Fusarium oxysporum* (Schlect emend. Snyder and Hansen) f. sp. *conglutinans* (Wr.). Races 1 and 5 have specificity for causing disease in cabbage (*Brassica oleracea* var. *capitata*), whereas race 2 has specificity for causing disease in radish (*Raphanus sativus*) (1, 17).

(A preliminary account of this work has appeared elsewhere [H. C. Kistler and S. A. Leong, J. Cell. Biochem., abstr. no. 9C, p. 170, 1985].)

MATERIALS AND METHODS

Fungal strains. All fungal strains used in this study (Table 1) were maintained in sterile soil at 4°C and grown on V-8 or potato glucose agar (24) at ambient laboratory temperature. Cultures for DNA extraction were grown on a rotary shaker in a rich medium (27) at 28°C.

Fungal DNA extraction. DNA was isolated by the method of Specht et al. (23). These preparations of total cellular DNA were sufficiently pure for use in some experiments. DNA was further purified by CsCl density gradient centrifugation (1.68 g of CsCl ml^{-1} and 120 μg of bisbenzamide ml^{-1}) as previously described (5). Samples were centrifuged in a Beckman 65 VTi rotor at 50,000 rpm for a least 16 h. The DNA separated into nuclear (lower) and mitochondrial (upper) fractions (see Results). Bands removed from gradients were extracted 4 times with CsCl-saturated isopropanol, dialyzed against STE (100 mM NaCl, 10 mM Tris hydrochloride, 1 mM disodium EDTA), ethanol precipitated, and dissolved in TE (10 mM Tris hydrochloride, 1 mM disodium EDTA [pH 8.0]).

Restriction enzyme analysis. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., International Biotechnologies, New England BioLabs, Inc., and Promega Biotech and used in accordance with the recommendations of the suppliers. Restriction endonuclease fragments were separated by horizontal agarose gel electrophoresis (0.5 to 1.5% agarose) in Tris-borate buffer (10) in a potential gradient of 1 to 5 V cm^{-1}. DNA was detected with ethidium bromide (0.5 μg ml^{-1}) stain by UV transillumination.

Cloning the 1.8-kilobase-pair (kbp) *Bgl*II fragment of pFOXC2. The fungal plasmidlike DNA pFOXC2 from strain PHW699 was separated from mitochondrial DNA (mtDNA) by preparative gel electrophoresis in 0.7% agarose–Tris-acetate buffer (10) at approximately 1 V cm^{-1}. The mtDNA and plasmid DNA were electrophoresed separately from the gel onto strips of DEAE paper (DE-81; Whatman, Inc.) and eluted from the paper with small volumes of 1 M NaCl–10 mM Tris–1 mM EDTA (pH 8.0). Plasmid DNA thus obtained was precipitated with ethanol, dissolved in TE, and digested to completion with *Bgl*II. This DNA was ligated with pUC12 (13) which had been linearized at the *Bam*HI site and treated with calf intestinal alkaline phosphatase (10). The ligation mixture was used to transform *Escherichia coli* TB1, a restriction-minus, modification-plus derivative of JM83 (13). Plasmid DNA of several Amp^r Lac^- transformants was analyzed by the alkaline lysis minipreparation method (10).

Isolation of mitochondria. Microconidia (2.8×10^9) of strain PHW699 were suspended in 1 liter of SC medium (21) and incubated for 18 h. Germinated spores were centrifuged and washed once with an isotonic medium (1.4 M $MgSO_4$, 50 mM sodium citrate [pH 5.8]) and then digested in the same buffer containing 1% (wt/vol) Novozym 234 (Novo Industries, Inc.). After 6 h of incubation at room temperature, the protoplasts were diluted 1:4 with the same buffer and har-

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TABLE 1. *F. oxysporum* f. sp. *conglutinans* strains

Strain	Race	Origin[a]	Host species
PHW2	1	Florida	*B. oleracea*
PHW81-4 (ATCC 52557)	1	Wisconsin	*B. oleracea*
PHW719	1	Hungary	*B. oleracea*
PHW722 (ATCC 9990)	1	ATCC	*B. oleracea*
PHW723 (ATCC 16600)	1	ATCC	*B. oleracea*
PHW744	1	Wisconsin	*B. oleracea*
PHW768	1	Wisconsin	*B. oleracea*
PHW777	1	Japan	*B. oleracea*
PHW684	5	California	*B. oleracea*
PHW811	5	California	*B. oleracea*
PHW699 (ATCC 58110)	2	Wisconsin	*R. sativus*
PHW724 (ATCC 16601)	2	South Carolina	*R. sativus*
PHW760	2	Germany	*R. sativus*
PHW779	2	Japan	*R. sativus*
PHW795 (CBS 262.50)	2	California	*R. sativus*
PHW796 (CBS 488.67)	2	CBS	*R. sativus*
PHW821	2	Taiwan	*R. sativus*
PHW1088	2	Japan	*R. sativus*

[a] ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

vested by flotation as follows. The diluted protoplasts were transferred to 50-ml centrifuge tubes, overlaid with 10 ml of 0.8 M mannitol–0.1 M Tris hydrochloride (pH 7.2), and centrifuged at 4,000 × *g* for 15 min in a Beckman 13.1 rotor maintained at 25°C. Protoplasts collected from the interface were washed twice with mannitol buffer by centrifugation at 1,000 × *g* for 5 min. Protoplasts were lysed by suspension in 0.29 M sucrose–10 mM Tris–5 mM EDTA (pH 7.6) on ice for 16 h, and nuclei and cell debris were removed by centrifugation at 1,000 × *g*. Mitochondria were collected by centrifugation at 20,000 × *g* and suspended in 1.75 M sucrose–10 mM Tris–5 mM EDTA (pH 7.6). One-half of this suspension was brought to 12 mM $MgCl_2$ and treated with 100 μg of DNase I ml^{-1} for 90 min at 4°C. To monitor nuclear DNA contamination, we did not treat the other half with DNase I. The organelles were again sedimented at 20,000 × *g* and lysed by incubation for 45 min at 37°C in a 0.44 M sucrose solution containing 1% sodium dodecyl sulfate and 500 μg of pronase ml^{-1}. Samples were extracted with phenol-chloroform and chloroform, ethanol precipitated, and dissolved in 30 μl of H_2O.

Electron microscopy. CsCl gradient-purified DNA was prepared for observation by the protein film technique and rotary shadowed as previously described (8). Specimens were observed in a JEM7 electron microscope.

Hybridization methods. DNA was fractionated by agarose gel electrophoresis, denatured, and transferred to nitrocellulose as previously described (12, 22). Prehybridization (1 to 16 h) and hybridization (16 to 48 h) were carried out in solutions of 50% formamide–5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7])–100 μg of heparin ml^{-1} (20) at 42°C. Blots were washed twice at 42°C for 1 h with 2× SSC–0.1% sodium dodecyl sulfate and rinsed briefly with 0.1× SSC–0.1% sodium dodecyl sulfate before being dryed. Papers were exposed at −70°C to Kodak X-Omat AR or OG-1 X-ray film with, respectively, Cronex Lightning-Plus or Lanex intensifying screens.

Nick translations of 0.1 to 0.2 μg of DNA with ^{32}P-labeled deoxynucleotides were carried out in 20 μl as described previously (12) to yield a specific activity of $>5 \times 10^7$ dpm μg^{-1}. The DNA probe was denatured by the addition of 0.3 N NaOH for 10 min, followed by neutralization with an appropriate volume of 1 M Tris hydrochloride (pH 7.4).

RESULTS

When total DNA from strain PHW699 was extracted and fractionated on CsCl-bisbenzamide density gradients, two major DNA-containing bands were observed. The larger, lower band contained high-molecular-weight DNA, which showed extreme complexity when digested with restriction enzymes and was presumed to be nuclear DNA (Fig.1). Southern hybridization experiments also showed that this DNA contained an 8.0-kbp *Sph*I sequence homologous to cloned nuclear rDNA from *Neurospora crassa* (data not shown). The smaller, upper band from the CsCl-bisbenzamide gradients also contained high-molecular-weight DNA. However, the restriction endonuclease digestion patterns obtained for this DNA were much simpler and yielded an estimated size of 43.4 ± 1.8 kbp, a value which is slightly lower than published values for mtDNA of *F. oxysporum* f. sp. *lycopersici* (11). Electron-microscopic analysis indicated that the mtDNA we isolated exists primarily as linearized molecules. Cosedimenting with this DNA fraction was a small double-stranded DNA of approximately 1.9 kbp (Fig. 1). Based on the intensity of staining with ethidium bromide, the 1.9-kbp element was estimated to exist at an equimolar ratio with mtDNA. Electron-microscopic analysis of this element revealed only linear molecules with a mean length of 0.64 ± 0.04 μm, $n = 13$ (data not shown). This length is consistent with an element of 1.9 kbp. The restriction endonuclease digestion patterns obtained for this DNA were also consistent with a linear molecule (Fig. 2). The symmetrical *Bgl*II sites near each terminus suggested the presence of terminal repeated sequences. This linear element has been designated pFOXC2 (plasmid *F. oxysporum* f. sp. *conglutinans* race 2).

A similarly sized linear molecule was also characterized from the race 1 strain PHW777. This element had a restric-

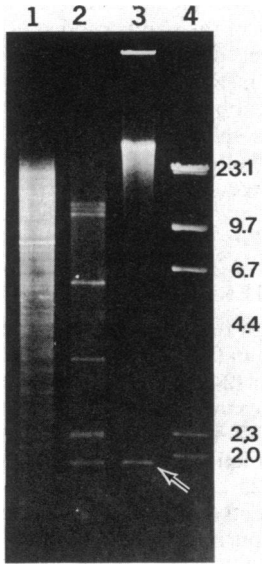

FIG. 1. Gel electrophoretic analysis of nuclear DNA and mtDNA fractions from CsCl-bisbenzamide-purified DNA of strain PHW699. DNA was electrophoresed on a 0.7% agarose gel and stained with ethidium bromide. Lanes show nuclear DNA (1)- or mtDNA (2)-containing fractions digested with restriction endonuclease *Sph*I or the undigested mtDNA fraction (3) containing plasmid pFOXC2 (arrow). λ DNA digested with *Hin*dIII (lane 4) is shown with the sizes of fragments given in kilobase pairs.

tion endonuclease cleavage map which was different from that of pFOXC2 and was designated pFOXC1 (Fig. 2).

Linear extrachromosomal DNAs from other sources generally have proteins covalently attached to their 5′ termini, making them insensitive to the 5′ → 3′ acting λ exonuclease. When DNA preparations of strain PHW699 containing mtDNA and pFOXC2 were incubated with λ exonuclease, pFOXC2 was insensitive, whereas the largely linearized mtDNA was slowly digested (Fig. 3). In contrast, both mtDNA and pFOXC2 were sensitive to exonuclease III, which acts in a 3′ → 5′ manner (data not shown).

Some fungal plasmids have been shown to share homology with mtDNA (4, 9) or nuclear DNA (28) or both, whereas others have homology with neither (14). To test for homology, we attemped to purify pFOXC2 from mtDNA by preparative gel electrophoresis and Sephacryl S-1000 gel filtration. However, because of the 20-fold greater mass of the mtDNA in these preparations, pFOXC2 was never isolated completely free of contaminating sheared mtDNA. To circumvent this problem, we digested DNA enriched for pFOXC2 with *Bgl*II and cloned it into the *Bam*HI site of phosphatase-treated pUC12. The predominant class of recombinant plasmids contained 1.8-kbp inserts that yielded the predicted pattern of restriction fragments after digestion with *Eco*RI. The *Bgl*II clone of pFOXC2 was designated pCK1 and contained all but about 100 base pairs of pFOXC2.

The symmetrical *Bgl*II sites of pFOXC2 (Fig. 2) suggested the presence of terminal repeated sequences. However, experiments to further test for such sequences have been inconclusive. For example, the large 3.5-kbp *Eco*RI fragment of pCK1 containing the 0.8-kbp *Eco*RI-*Bgl*II fragment of pFOXC2 (and the 2.7-kbp vector) shared little or no homology with the smaller 1.0-kbp *Eco*RI fragment of pCK1 containing the 1.0-kbp *Eco*RI-*Bgl*II fragment of pFOXC2 (Fig. 4). Thus, if terminal inverted repeats exist in pFOXC2, they cannot extend much further than the *Bgl*II sites. This might explain our failure to observe "stem and loop" structures by electron microscopy of pFOXC2 after denaturation and renaturation of the DNA. Such structures are unstable and not detectable when terminal repeated sequences are short (3).

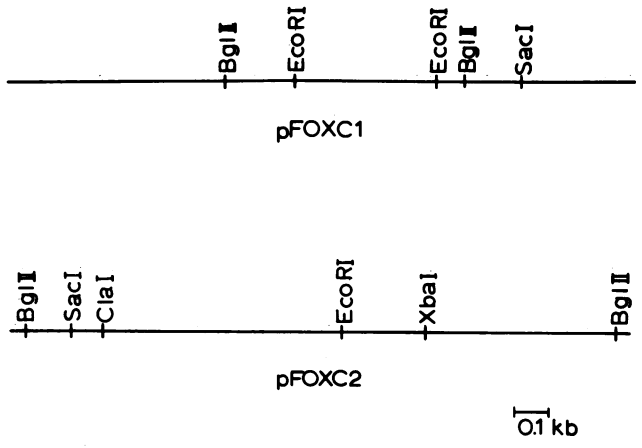

FIG. 2. Restriction endonuclease maps of 1.9-kbp elements, designated pFOXC1 and pFOXC2, derived from race 1 strain PHW777 and race 2 strain PHW699, respectively. Maps were deduced from single and double endonuclease digestions. The recognition sites for *Bam*HI, *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I, and *Xho*I are not present in pFOXC1. The element pFOXC2 lacks sites for *Bam*HI, *Hin*cII, *Hin*dIII, *Pst*I, *Sph*I, and *Xho*I. kb, Kilobase pairs.

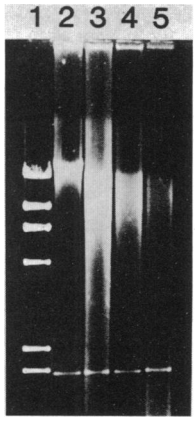

FIG. 3. Gel electrophoretic analysis of λ exonuclease digestion of DNA from mtDNA fractions of strain PHW699. The DNA, purified by CsCl-bisbenzamide gradient centrifugation, was incubated with 4 U of λ exonuclease at 37°C in buffer recommended by the supplier (New England BioLabs). One unit of λ exonuclease is defined as the amount generating 10 nmol of acid-soluble nucleotide in 30 min at 37°C. The reaction was terminated by heating at 80°C for 10 min after incubations for 0 (lane 2), 1 (lane 3), 2 (lane 4), or 3 (lane 5) h. Digests were electrophoresed on an 0.8% agarose gel along with *Hin*dIII-digested λ DNA (lane 1) and stained with ethidium bromide.

When pCK1 was used as a probe against nuclear DNA and mtDNA of strain PHW699, it was homologous only to the

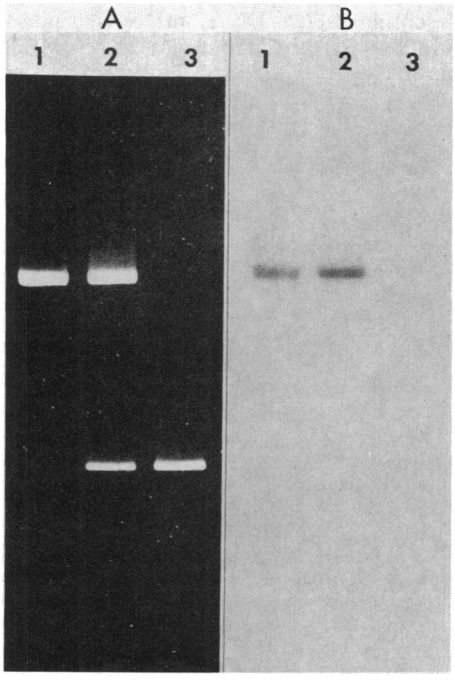

FIG. 4. Two fragments from *Eco*RI digestion of plasmid pCK1. These fragments were separated on 0.7% agarose gels and isolated as described in the text. Total *Eco*RI digests of pCK1 or individually isolated fragments were separated on a 0.7% agarose gel. Lanes: 1, 3.5-kbp fragment (0.4 μg); 2, *Eco*RI-digested pCK1 (0.4 μg); 3, 1.0-kbp fragment (0.1 μg). (A) Ethidium bromide-stained gel. (B) Autoradiogram of Southern transfer of gel probed with the ^{32}P-labeled 3.5-kbp fragment.

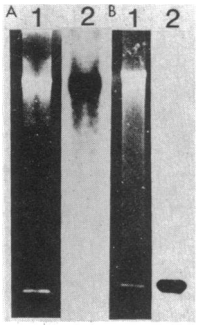

FIG. 5. Southern hybridization analysis of mtDNA and plasmid-like DNA from strain PHW699. Lanes: 1, ethidium bromide-stained gels of 0.5 μg of DNA from mitochondrial fractions of strain PHW699; 2, autoradiograms of Southern transfers of these gels hybridized with ^{32}P-labeled, gel-purified, high-molecular-weight mtDNA from strain PHW699 (A) or ^{32}P-labeled pCK1 (B).

1.9-kbp band corresponding to pFOXC2 (Fig. 5). The nuclear DNA isolated in the manner described above also contained a small amount of homology to pFOXC2 (data not shown) but only at a band corresponding to 1.9 kbp, indicating that this DNA contained a small amount of contaminating pFOXC2 DNA. When mtDNA was isolated free of pFOXC2 and used as a probe, no homology between mtDNA and pFOXC2 was observed (Fig. 5).

To confirm the mitochondrial location of pFOXC2, we isolated mitochondria and treated them with DNase I to eliminate contaminating nuclear DNA. After the removal of DNase I, the organelles were lysed, and the DNA was extracted. Equal quantities of this DNA were probed with DNA representing nuclear DNA, mtDNA, or plasmid DNA (Fig. 6), as follows: (i) cloned nuclear rDNA (pMF2) from *N. crassa* (18), (ii) cloned mitochondrial large rDNA containing fragment *Pst*3 from *Podospora anserina* (29), or (iii) pFOXC2 DNA (pCK1). The DNA from isolated mitochondria contained sequences with extensive homology to mtDNA and pCK1, and this homology was not reduced by DNase treatment. Hybridizing material was detected with the cloned nuclear rDNA from *N. crassa*, but this hybridization was considerably reduced by the DNase wash. From these data, we suggest that pFOXC2 exists in the mitochondria, but we do not exclude the possibility that it may also exist in the cytoplasm or nucleus, as some hybridization to a 1.9-kbp sequence was observed when the nuclear DNA fraction was probed with pCK1 (Fig. 6).

Additional strains of *F. oxysporum* f. sp. *conglutinans* race 2, race 1, and race 5 were examined for the presence of the 1.9 kbp plasmidlike DNA. All strains examined had similarly sized elements in the mtDNA fraction of CsCl-bisbenzamide gradients (Fig. 7 and 8). However, only the elements from the race 2 strains were homologous to the cloned race 2 plasmid pCK1. Likewise, all 1.9-kbp elements from races 1 and 5 contained homologous DNA sequences. When the plasmidlike DNA of race 1 strain PHW81-4 was used as a probe, 1.9-kbp bands were detected only in mtDNA from race 1 and 5 isolates (Fig. 8). A small amount of hybridization was also observed to a band of the size of a dimeric plasmid (Fig. 6, 7, and 8) as well as mtDNA (Fig. 8). This latter hybridization was not surprising, since the nick-translated plasmid-containing fraction in Fig. 8 was contaminated with sheared mtDNA. The specificity found in the hybridization analysis was also supported by restriction endonuclease cleavage patterns obtained for these elements (Fig. 2). For example, all race 2 plasmidlike DNAs were cleaved once with *Eco*RI at the same apparent location as pFOXC2, whereas all race 1 and 5 plasmidlike DNAs were cut twice with *Eco*RI at the same apparent location as pFOXC1.

DISCUSSION

This paper is the first description of linear plasmidlike DNAs in the plant pathogenic fungus *F. oxysporum* f. sp.

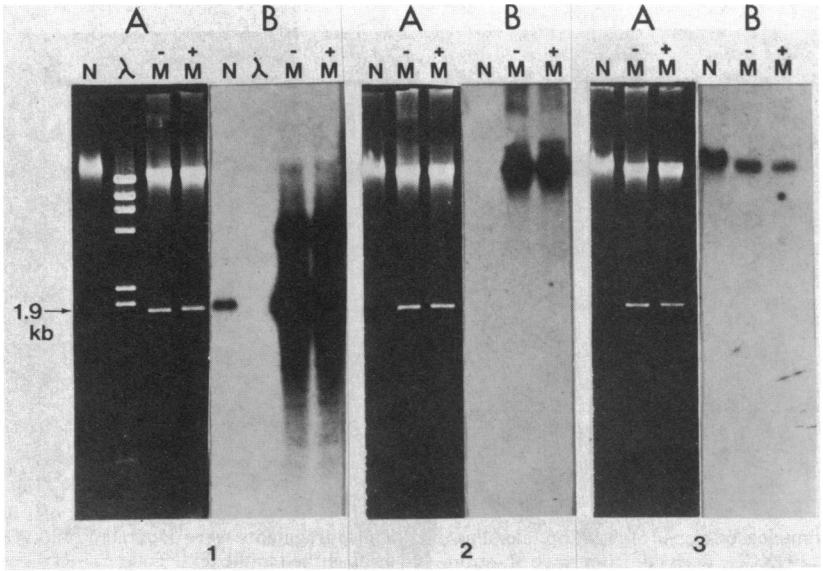

FIG. 6. Gel electrophoretic and Southern hybridization analyses of DNA isolated from intact mitochondria. Equal quantities (10 μl) of mitochondrial DNA (lanes M) derived from isolated mitochondria, treated (+) or untreated (−) with DNase I prior to extraction of DNA, were separated on 0.7% agarose gels with *Hin*dIII-digested λ DNA as a size marker (lane λ). Subpanel A of each panel shows ethidium bromide-stained gels, and subpanel B shows autoradiograms of Southern transfers of gels probed with pCK1 (panel 1), *Pst*3 (panel 2), or pMF2 (panel 3). Control DNA (lane N) contains 0.2 μg of the nuclear DNA fraction of strain PHW699. kb, Kilobase pairs.

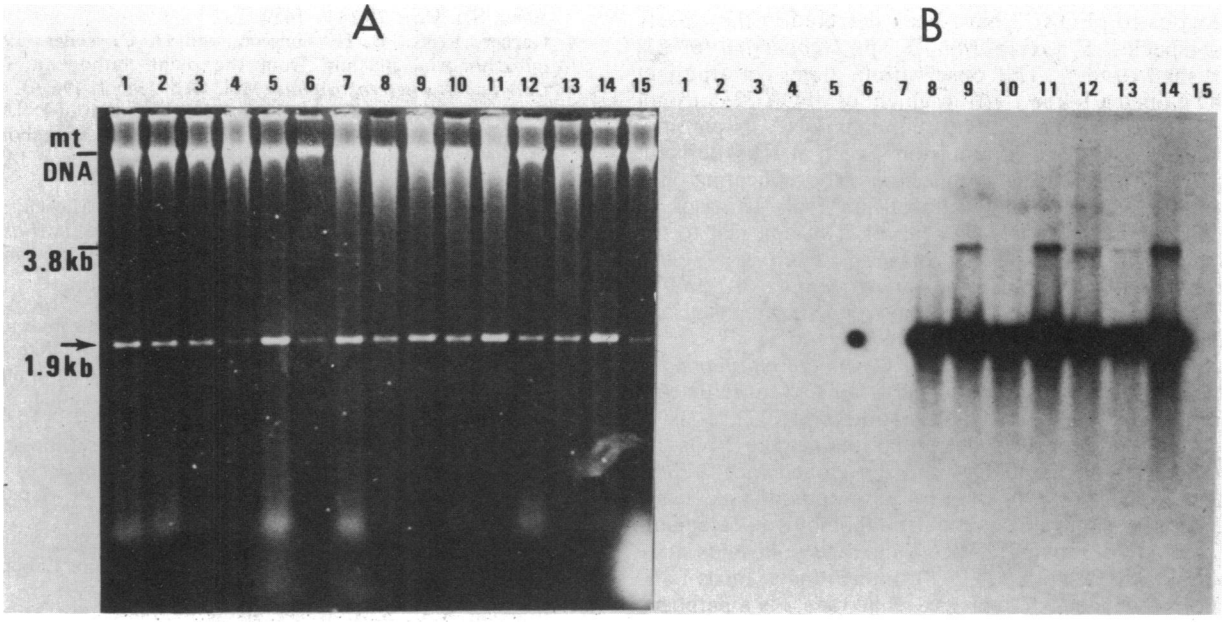

FIG. 7. Gel electrophoretic and Southern hybridization analyses of mtDNA fractions from CsCl-bisbenzamide gradients. DNA from races 1, 2, and 5 of *F. oxysporum* f. sp. *conglutinans* was electrophoresed on a 0.7% agarose gel and detected with ethidium bromide (A). An autoradiogram (B) of Southern transfer of the gel was probed with ^{32}P-labeled pCK1. Lanes show DNA from the following strains: 1, PHW2; 2, PHW722; 3, PHW723; 4, PHW768; 5, PHW777; 6, PHW684; 7, PHW811; 8, PHW699; 9, PHW724; 10, PHW760; 11, PHW795; 12, PHW796; 13, PHW821; 14, PHW1088; and 15, PHW719. Approximately 0.5 μg of DNA was loaded in each lane. The arrow shows the position of the 1.9-kbp plasmidlike DNA bands. kb, Kilobase pairs.

conglutinans. The linear double-stranded DNA molecules appear to be similar to linear plasmids described for other filamentous fungi (26) in that they have blocked 5′ termini and are localized in the mitochondria. However pFOXC1, pFOXC2, and the other *F. oxysporum* f. sp. *conglutinans* 1.9-kbp molecules are the smallest reported linear DNAs. Although similarly sized elements were found in all strains of this fungus, no extrachromosomal elements or sequences

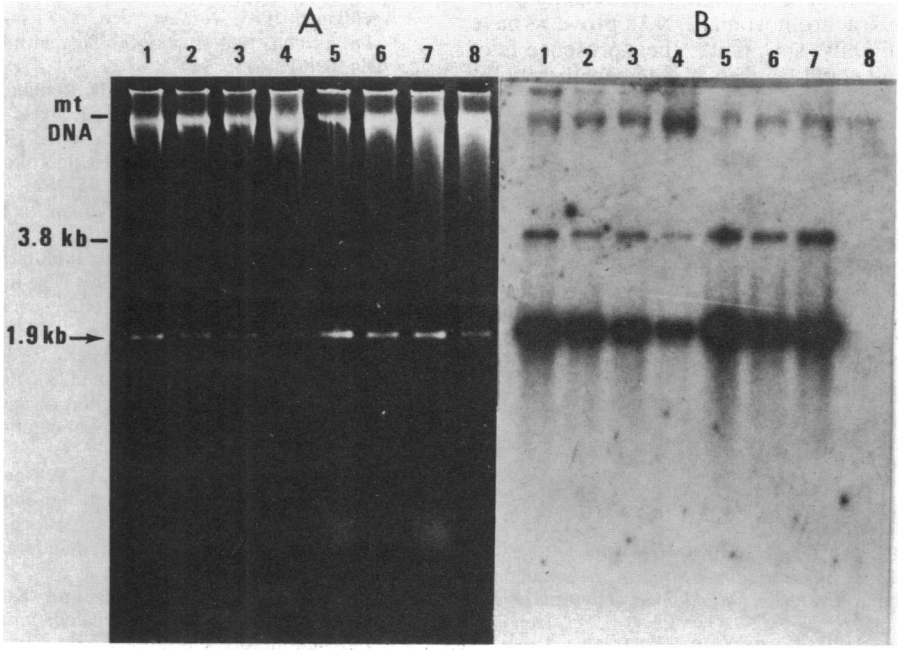

FIG. 8. Gel electrophoretic and Southern hybridization analyses of CsCl-bisbenzamide gradient-purified mtDNA fractions of strains representing races 1, 2, and 5. (A) Ethidium bromide-stained 0.7% agarose gel; (B) autoradiogram of Southern transfer of the gel probed with the ^{32}P-labeled, gel-purified, plasmidlike DNA from race 1 strain PHW81-4. Lanes show DNA from the following strains: 1, PHW2; 2, PHW722; 3, PHW723; 4, PHW768; 5, PHW777; 6, PHW684; 7, PHW811; and 8, PHW699. Approximately 0.5 μg of DNA was loaded per lane. The arrow shows the position of the 1.9-kbp plasmidlike DNA bands. kb, Kilobase pairs.

homologous to pFOXC2 have been detected in the closely related species *F. oxysporum* f. sp. *lycopersici* (6, 11; unpublished results). This observation, along with the fact that the cloned 1.8-kbp *Bgl*II fragment of pFOXC2 does not have sequences homologous to either mtDNA or chromosomal DNA from the strain from which it was derived, suggests that pFOXC2 is an autonomously replicating plasmid or virus unessential for fungal survival. The lack of homology of pCK1 to mtDNA places it in contrast to the circular fungal mitochondrial plasmids from *Aspergillus amstelodami* (9), *Cochliobolus heterostrophus* (4), and *P. anserina* (15, 28), all of which may represent excised and amplified elements of the mitochondrial genome.

Races of *Fusarium oxysporum* f. sp. *conglutinans* are defined by the ability of pathogenic strains to cause disease in some plant species but not in others (1, 17). It is of considerable interest then that all strains of race 2 possess plasmidlike DNAs homologous to the cloned race 2 *Bgl*II fragment from pFOXC2, whereas all strains of race 1 and race 5 possess similarly sized but nonhomologous elements (Fig. 7 and 8). Moreover, all race 1 and race 5 plasmids share homology with each other (Fig. 8). Interestingly, races 1 and 5 are both pathogens of cabbage, while race 2 is a pathogen of radish. Race 5 is distinguished from race 1 by its ability to attack cabbage lines carrying monogenic dominant type A resistance (17). Since these plasmidlike DNAs are host-specific, they may carry genes that determine host specialization. Alternatively, these elements may be present in isolates of wide geographic distribution because they have arisen as clones of race progenitor strains, each of which possess unique plasmidlike DNAs.

We are presently extending our analysis to other isolates and races of this pathogen to determine if the correlation of race and host-specific plasmidlike DNAs is maintained. In addition, we wish to determine whether these elements contain open reading frames that are transcribed and translated and, if so, the biological function of these gene products. Even if these extrachromosomal DNAs prove to have no role in determining host specificity, their presence is of taxonomic interest and could be used as a diagnostic tool for judging the presence of specific pathogen races.

ACKNOWLEDGMENTS

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