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In Vivo Rearrangement of Foreign DNA by *Fusarium oxysporum* Produces Linear Self-Replicating Plasmids

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Particular combinations of fungal strains and transformation vectors allow for fungal rearrangement of normally integrative plasmids, resulting in the creation of linear self-replicating plasmids in *Fusarium oxysporum*. The rearrangement results in the addition of fungal DNA, including telomere consensus sequences, to plasmid termini. The mechanism by which this rearrangement occurs is unclear, but it has similarities to extrachromosomal gene amplification. A DNA fragment which allows for linear autonomous replication upon reintroduction to the fungus was subcloned and sequenced. This DNA sequence contains the repeated telomeric sequence TTAGGG flanked by a region of twofold symmetry consisting primarily of pUC12 DNA. Isolation and identification of this sequence is the first step toward development of vectors that function as artificial chromosomes in filamentous fungi. This sequence was shown to promote autonomous replication and enhance transformation in several strains of *F. oxysporum*, *Nectria haematococca*, and *Cryphonectria parasitica*.

We have discovered a fungus-vector combination which promotes in vivo production of self-replicating plasmids. Our original goal was to produce an efficient transformation system for the plant pathogenic fungus *Fusarium oxysporum* Schlecht. We initially sought to improve our fungal vector by incorporating sequences from a linear *Fusarium* plasmid to determine if the plasmid sequences would promote autonomous replication. Similar experiments have been performed in *Neurospora crassa* by using mitochondrial plasmids from *Neurospora intermedia* LaBelle (15, 29). The chimeric vector we constructed did not, without modification by the host, replicate autonomously in *F. oxysporum* transformants, nor did it increase transformation frequency. This vector appeared to transform the fungus primarily by integration into the chromosomal DNA, as described for other vectors previously used with *F. oxysporum* (13), and is common in filamentous fungi (7). Upon examination of transformants in other *F. oxysporum* strains, several were found to contain autonomously replicating plasmids similar but not identical to the transforming vector. In these same transformants, no detectable vector sequences were found incorporated into the chromosomal DNA. Since these plasmids were maintained separately from the chromosomal DNA, they must contain a *Fusarium* autonomously replicating sequence (ARS).

ARSs from *Saccharomyces cerevisiae* have been defined by their ability to allow bacterial plasmids to freely replicate in yeast cells (33). The consequence of adding ARSs to integrative yeast vectors is the production of yeast replicating plasmids that have the properties of high-efficiency plasmid transfer and high plasmid copy number. Some yeast ARSs have been shown to be origins of DNA replication (4, 11, 28). Yeast ARSs have not been found to replicate in filamentous fungi (7). The advantages of yeast replicating plasmid vectors have prompted the search for ARSs from filamentous fungi in order to improve transformation systems in these organisms (9, 20, 21, 30). Recently, an ARS was cloned from the basidiomycetous fungus *Ustilago maydis* (31). This sequence, when incorporated into an integrative vector, allowed for autonomous replication and increased the frequency of *U. maydis* transformation several thousandfold. We have isolated a DNA fragment from an autonomously replicating plasmid from the ascomycetous fungus *F. oxysporum*. This fragment increases transformation several thousandfold and allows for autonomous replication but is not similar to the ARS in *U. maydis*.

The autonomously replicating plasmids detected in the *F. oxysporum* transformants were of interest for three reasons. First, they were produced in the fungus by rearrangement of the original integrative vector by an undetermined mechanism. Second, the modification of the original vector added relatively little new DNA, but sequences which were added contained functional ARS and telomeric elements. To our knowledge, these are the first characterized sequences to function as telomeres isolated from a filamentous fungus. Third, the production of these autonomously replicating plasmids gave the opportunity to construct high-transformation efficiency, autonomously replicating vectors. These vectors may prove to be very useful tools for future research in *F. oxysporum* and other filamentous fungi. This paper describes the characterization of linear autonomously replicating plasmids produced in vivo by *F. oxysporum* during transformation.

MATERIALS AND METHODS

Fungal strains. *F. oxysporum* f. sp. *lycopersici* 73 was donated by Felice Cervone, Plant Biology Department, University of Rome—"LaSapienza," Italy. *F. oxysporum* f. sp. *conglutinans* PHW808, PHW777, PHW722, and PHW719 and *F. oxysporum* f. sp. *raphani* PWH699 were supplied by Paul H. Williams, Department of Plant Pathology, University of Wisconsin—Madison. *F. oxysporum* f. sp. *pisi* 247 and *Nectria haematococca* T2 were provided by Hans VanEtten, Cornell University, Ithaca, N.Y. *Magnaporthe grisea* PH-II-82 was obtained from Masatoki Taga, Sakata Seed Co., Yokohama, Japan. *Cryphonectria parasitica* EP155 was donated by Dennis Fulbright, Department of Plant Pathology, Michigan State University, East Lansing.

Fungal transformations. Conidia from *Fusarium* strains were isolated from 5-day-old cultures grown in 50 ml of

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potato dextrose broth (PDB; Difco Laboratories, Detroit, Mich.) shaken at 150 rpm at room temperature. Conidia from *C. parasitica* were isolated from 2-week-old potato dextrose agar (PDA) cultures by washing the surface with sterile distilled water. Both the liquid cultures and plate washes were filtered through two layers of sterile cheesecloth, and the conidia were pelleted by centrifugation at 1,500 × *g* for 5 min. The conidia were washed twice in sterile distilled water. Approximately 1×10^9 conidia were germinated in 50 ml of PDB by shaking for 12 to 14 h. The germinated conidia were collected by centrifugation at 1,500 × *g* for 5 min in sterile tubes. The germinated conidia were washed twice with OB (1.4 M $MgSO_4$, 50 mM sodium citrate [pH 5.8]). The germinated conidia were digested with 10 ml of OB–2% NovoZym 234 (Novo BioLabs, Danbury, Conn.) at room temperature for 1 to 4 h. When greater than 90% of the protoplasts were released, they were filtered through two layers of sterile cheesecloth and then centrifuged at 1,500 × *g* at room temperature for 30 min. The protoplasts were gently aspirated from the top of the suspension and washed twice with TB (1.0 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris chloride [pH 7.4]). The protoplasts were suspended at 5×10^8 protoplasts per ml in TB–6% polyethylene glycol 4000 –1% dimethyl sulfoxide. The protoplasts were stored as aliquots at −70°C.

The protoplasts were transformed by thawing a frozen suspension and adding a nuclease inhibitor, aurintricarboxylic acid, to a final concentration of 2 mM (24). Vector DNA in 10 μl of TE (18) was added to 200 μl of the protoplast suspension and incubated for 30 min on ice. A solution of 60% polyethylene glycol 4000–50 mM $CaCl_2$ was added in 2 200-μl and 1 800-μl volumes, with gentle mixing between each addition, and was incubated for 30 min on ice. Then 2 ml of 1.0 M Sorbitol–0.5× PDB was added and gently mixed. The protoplasts were pelleted by centrifuging at 1,500 × *g* for 5 min at room temperature. The pellet was suspended in 1 ml of 1.0 M Sorbitol–0.5× PDB. Dilutions of the protoplasts were then spread on 1.0 M Sorbitol–0.1× PDA plates which had been freshly poured in two layers. The bottom layer contained 200 μg of hygromycin B per ml, which slowly diffused into the top layer. The plates were incubated at 24°C for 7 to 10 days. The first transformants appeared by day 3.

DNA isolations. Large-scale nuclear and mitochondrial DNA isolations have been previously described (14). DNA "minipreps" from fungi were obtained from strains grown in 15 ml of PDB with 10 μg of hygromycin B per ml. The unshaken mycelium was harvested after 3 to 5 days of growth by pouring through cheesecloth. The mycelium was pressed dry between paper towels and frozen at −70°C in a Microfuge tube (Beckman Instruments, Inc., Fullerton, Calif.). The frozen mycelium was lyophilized overnight and then ground to a fine powder with a wooden stick in the Microfuge tube. A 500-μl volume of lysis buffer (100 mM Tris chloride [pH 8.0], 50 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 1% sodium dodecyl sulfate) was added and mixed. The solution was incubated at 65°C for 10 min. The samples were cooled on ice, and 250 μl of cold 5.0 M potassium acetate was added and mixed, and the samples were returned to ice for 20 min. The samples were centrifuged in a Microfuge at top speed for 10 min. The supernatant was collected, and the RNA was removed by incubation with 50 μg of heat-treated RNase per ml for 30 min at 37°C. Proteinase K was added to a final concentration of 100 μg/ml and incubated for 10 min at 37°C. The samples were extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ethanol at room temperature for 2 min. The samples were centrifuged in a Microfuge at top speed for 15 min. The pellets were washed with 1 ml of 70% ethanol and centrifuged for 5 min. The pellets were dried and then suspended in 50 μl of TE buffer and stored at 4°C.

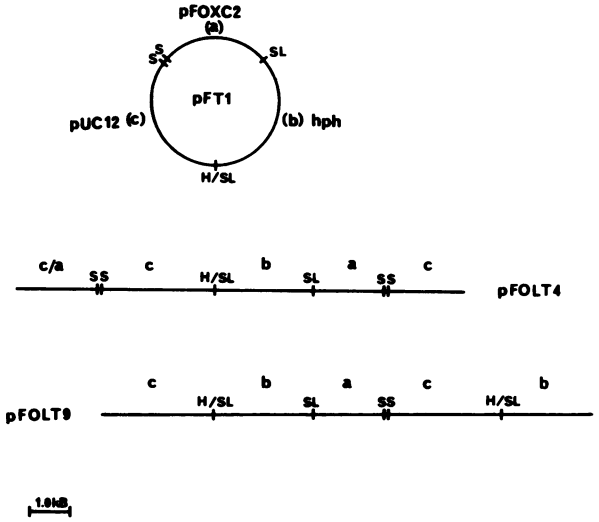

FIG. 1. Partial restriction site maps of pFT1, pFOLT4, and pFOLT9. Only the location of restriction endonuclease sites *Sst*I (S), *Hin*dIII (H), and *Sal*I (SL) are shown. The circular plasmid pFT1 was the transformation vector from which the autonomous plasmids arose. Components of pFT1 are the 1.8-kb fragment from pFOXC2 (a), the *hph* gene (b), and pUC12 (c). The linear plasmids pFOLT4 and pFOLT9 were produced in vivo by the fungus from pFT1. Maps were produced by digesting total DNAs from the fungal transformants with the appropriate enzymes, separating DNAs on an agarose gel, blotting, and probing with ^{32}P-labeled pFT1. The junction sites between pFT1 sequences on pFOLT4 and pFOLT9 and the inverted repeats near the termini remain to be determined.

Recovery of plasmids from transformed fungi. The linear plasmids were eluted from bands after separation of undigested fungal DNA on a 0.7% agarose gel in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) at 1 V/cm. The recovered DNA in its linear form or a circularized form was used to transform competent *Escherichia coli* DH5α. Three methods were attempted to circularize the plasmid. The first method attempted to ligate the unmodified termini with only T4 ligase. The second method involved incubation of the plasmid with the Klenow fragment of DNA polymerase followed by treatment with T4 ligase. Plasmids recovered by these methods were indistinguishable from the original transforming vector pFT1 (see Results; Fig. 1). The final method, which allowed recovery in *E. coli* of the sequences modified in vivo within the isolated linear plasmid, involved initial digestion with S1 nuclease. The linear plasmid was incubated with 4 U of S1 nuclease in its reaction buffer [30 mM sodium acetate (pH 4.6), 150 mM NaCl, 1 mM $Zn(OAc)_2$, 5% (vol/vol) glycerol] at 37°C for 30 min. The reaction was stopped by bringing the solution to 25 mM EDTA and 100 mM Tris chloride, pH 8.0. Yeast tRNA (1.0 μg) was added, and the sample was precipitated with 2 volumes of ethanol. The sample was placed at −70°C for 30 min and then centrifuged in a Microfuge at top speed for 15 min. The pellet was washed with 70% ethanol, dried, and suspended in 7 μl of water, to which was added 1 μl of 2 mM deoxynucleoside

triphosphates, 1 μl of 10× Klenow buffer (100 mM Tris chloride [pH 7.5], 500 mM NaCl), and 1 μl (1 U) of Klenow fragment of DNA polymerase. The reaction was incubated at 37°C for 15 min. The sample was then self-ligated by adding 4 μl of water, 4 μl of 5× ligation buffer (250 mM Tris chloride [pH 7.6], 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25% [wt/vol] polyethylene glycol 8000), and 2 μl (2 U) of T4 ligase. The reaction was carried out at room temperature for 4 h and precipitated by the addition of 2 volumes of ethanol and centrifugation in a Microfuge. The pellet was washed with 70% ethanol and dried. The plasmid was suspended in TE and used to transform DH5α. A plasmid recovered in this way was designated pFOLT4R4.

Examination of stability of pFOLT4. To determine if the autonomously replicating plasmid pFOLT4 was stably maintained during formation of conidia, cultures of the transformants 73/FT1/T4 (strain 73 containing pFOLT4) and 73/FT1/T2 (strain 73 containing pFT1 integrated into the chromosomal DNA) were grown in PDB with or without hygromycin B (10 μg/ml) selection for 3 days. Conidia were isolated by filtering the cultures through sterile cheesecloth and centrifuging the filtrates at 2,000 × *g* for 5 min. The conidia were washed twice with sterile distilled water, and dilutions were plated on PDA plates. The plates were incubated at room temperature overnight. Germinated conidia were then transferred to PDA plates containing 50 μg of hygromycin B per ml.

To test stability during vegetative growth, the transformants were grown on PDA plates with or without hygromycin B (100 μg/ml) selection. After 2, 4, 7, and 10 days, eight 1-mm^3 plugs were transferred from the growing margin to PDA plates containing 100 μg of hygromycin B per ml. After 10 days, plugs were removed from the interior of the colonies at locations corresponding to where the original plugs were removed, and the plugs transferred to selection plates.

Miscellaneous techniques. Subcloning DNA fragments, agarose gel electrophoresis, hybridizations, and other standard procedures have been described previously (18). Screening subclones for ARS activity was performed by ethanol precipitation of the ligation mixes suspended in TE and by using half of the volume for the transformation procedure described above. Ligation mixes which gave a high transformation frequency in fungi were then used to transform DH5α, and the plasmids from subsequent clones were isolated by the alkaline lysis procedure (18).

DNA probes were made from linearized DNA fragments, some of which were isolated from agarose gels, and all were labeled with ^{32}P by using a random primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the instructions of the manufacturer.

DNA samples (<25 μl) for slot blot hybridization were denatured with 0.4 M NaOH prior to binding to nitrocellulose under vacuum in a slot blot apparatus (Bethesda Research Laboratories, Gaithersburg, Md.). Hybridizations were carried out under previously described conditions (14). To estimate the copy number of free plasmid in fungal cells, DNAs from fungal minipreps were hybridized to ^{32}P-labeled pFT1 (see Results) and compared with a dilution series of pFT1 standards on the same filter. Relative intensities were determined by using a scanning densitometer on X-ray film exposed to filter hybridizations.

DNA sequencing was performed at the University of Florida DNA sequencing core facility by Ernesto C. Almira on a Genesis 2000 DNA analyzer. Some sequences which could not be obtained from this facility were obtained by using a Sequenase sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) according to the instructions of the manufacturer. The sequences were resolved on an 80-cm 6% polyacrylamide–7 M urea–1× TBE (18) gel run at 50°C and 2,500 V.

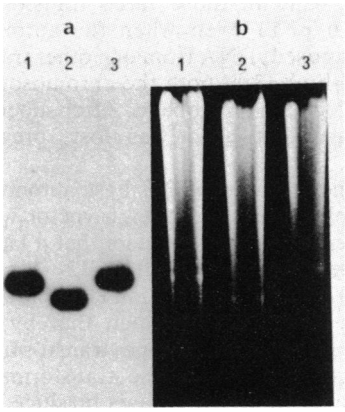

FIG. 2. Detection of autonomously replicating plasmids in DNAs isolated from transformants. Total DNAs were isolated from *F. oxysporum* f. sp. *lycopersici* 73 transformants, separated on a 0.7% agarose gel with TAE buffer, and stained with ethidium bromide (b). DNAs separated in this manner were blotted and probed with ^{32}P-labeled pFT1 (a). The labeled pFT1 hybridized only to low-molecular-weight bands approximately 10.8, 10.0, and 11.4 kb. Shown are DNAs from transformants T4 (lanes 1), T7 (lanes 2), and T9 (lanes 3). The autonomously replicating plasmids were designated pFOLT4, pFOLT7, and pFOLT9.

RESULTS

Identification of autonomous plasmids induced by transformation. The fungal vector pFT1 (Fig. 1) was constructed as follows: a 1.8-kilobase (kb) fragment from pFOXC2, the linear mitochondrial plasmid endogenous to *F. oxysporum* f. sp. *raphani*, was cloned into pUC12, producing pCK1 (14). A 2.4-kb *Sal*I fragment from pDH25 (6), which contains the bacterial hygromycin B phosphotransferase gene (*hph*) driven by the *Aspergillus nidulans trpC* promoter, was ligated into the single *Sal*I site of pCK1. The resulting plasmid, pFT1, was used to transform *F. oxysporum* strains to determine the effect of the pFOXC2 sequences on transformation frequency and the fate of the vector in the cell. By using optimized conditions, transformation of *F. oxysporum* strains with pFT1 gave a transformation frequency of approximately 10 transformants per μg of vector.

F. oxysporum f. sp. *lycopersici* 73 was the first strain observed to produce autonomous plasmids after being transformed with pFT1. The transformation efficiency of strain 73 was atypical, with a low frequency of approximately one transformant per 4 μg of vector. Eleven transformants of strain 73 were obtained from several transformation experiments with pFT1, and their DNAs were examined to determine the fate of the vector. Total DNA from nontransformed strain 73 did not hybridize to pFT1. DNAs from 7 of the 11 transformants hybridized with pFT1 only at the high-molecular-weight band corresponding to chromosomal DNA. These results are similar to data obtained from transformation of other *F. oxysporum* strains by using other vectors (13). However, three of the transformants (T4, T7, and T9) contained low-molecular-weight DNA bands, not present in the original strain 73, which hybridized to pFT1 (Fig. 2). The

chromosomal band in those three transformants did not hybridize with pFT1 even when the autoradiograph was greatly overexposed. DNA from one other transformant (T1) hybridized with pFT1 at both the chromosomal DNA band and a low-molecular-weight band. After subculturing T1, the low-molecular-weight band was lost, preventing further study.

To determine if production of these autonomous plasmids required the pFOXC2 sequences, a vector was constructed which was identical to pFT1 except that it lacked the 1.8-kb pFOXC2 insert. This vector (pHRC12) was used to transform strain 73. The transformation efficiency of strain 73 by pHRC12 was slightly higher than that by pFT1, with a frequency of approximately one transformant per μg of vector. DNAs from 13 of these transformants were analyzed. Twelve of the transformants produced with pHRC12 contained the vector incorporated into high-molecular-weight DNAs. One transformant (T8) contained an autonomous plasmid. Therefore, the pFOXC2 sequences were not a necessary factor in formation of autonomous plasmids, but they may increase the frequency of autonomous plasmid formation.

The stability of the autonomous plasmids was examined by growing the transformants of strain 73 in the presence and absence of the antibiotic hygromycin B. Conidia produced in liquid cultures were tested for maintenance of plasmid-encoded drug resistance. The transformants T4, from which the autonomously replicating plasmid pFOLT4 arose, and T2, containing pFT1 integrated into the chromosomal DNA, were used in this study. Fifty germinated conidia were transferred to hygromycin B-containing plates from each transformant. Conidia isolated from cultures grown under nonselective conditions produced 50 colonies for T2 and 14 for T4. All the conidia from both T2 and T4 were hygromycin B resistant when previously grown in the presence of the antibiotic. This indicated that the autonomously replicating plasmids were not stable during conidia formation without selection, but the integrated vector was stably transferred under both conditions.

The loss of autonomous plasmids during vegetative growth without selection was also detected. Mycelium grown on agar medium without selection maintained resistance for up to 4 days. After 7 days, only hygromycin-sensitive subcultures could be obtained from the growing margin of the colony. The growing mycelium lost the plasmid, but the mycelium isolated from the older parts of the colony (which grew during the first 4 days) still retained the plasmid. It is not known if the plasmid is retained in the mycelium or in the conidia formed in that portion of the colony. Therefore, autonomous plasmids are unstable without selection, and either cells containing the plasmids are unable to compete with cells free of the plasmid or the plasmid is inefficiently transferred or partitioned during cell division.

Characterization of the pFT1-derived autonomous plasmids. DNAs from strain 73 transformants T4, T7, and T9 were examined to determine the structural form of their autonomous plasmids, called pFOLT4, pFOLT7, and pFOLT9, respectively. Total DNA was isolated on cesium chloride gradients in the presence of ethidium bromide or bisbenzimide. In both types of gradients, the autonomous plasmids banded at the same position as nuclear chromosomal DNA. This indicated that the autonomous plasmids differ from the linear mitochondrial plasmid found in *F. oxysporum* from which pFT1 was partially derived. Mitochondrial plasmids band with the mitochondrial DNA fraction in the presence of bisbenzimide (14). The banding of the autonomous plasmids with nuclear DNA in CsCl-ethidium bromide gradients suggested that the plasmids were linear.

Restriction endonuclease maps of pFOLT4 and pFOLT9, made by digesting total DNA and visualizing the banding pattern by hybridization with labeled pFT1, were also linear. For simplicity, an example of a partial map with only three enzymes is shown (Fig. 1). Figure one shows the restriction endonucleases which divide pFT1 into its three components: pUC12, the *hph* gene, and pFOXC2 sequences. Examination of the maps of pFOLT4 and pFOLT9 suggests that they have been derived from a tandemly repeated pFT1 precursor. Each plasmid contained one complete copy of pFT1, with additional portions of the original plasmid at the ends. The sequences nearest to the ends of the plasmids were not identical to pFT1, as determined by the loss of expected restriction endonuclease sites. The plasmid pFOLT7 from transformant T7 appeared to have been greatly rearranged with respect to pFT1, with the loss of many restriction sites. Hybridizations of restriction endonuclease digests of pFOLT7 with the *hph* gene fragment of pFT1 indicated that much of this plasmid contained these sequences. Since the mapping of these plasmids utilized total DNA, creating a complete map of pFOLT7 with pFT1 as a probe was not possible. A complete map of pFOLT7 will be made if it retained enough of the pUC12 sequences to be recovered. Complete maps of some other autonomous plasmids produced in strain 73 or in *F. oxysporum* f. sp. *pisi* 247 are also yet to be determined. Results with a small number of restriction enzymes on a few of these plasmids indicated a pattern similar to that of pFOLT4 and pFOLT9.

Plasmids pFOLT4, pFOLT7, and pFOLT9 could be detected in agarose gels by ethidium bromide staining if the DNA isolation procedures did not excessively shear the chromosomal DNA. The copy number of the autonomous plasmids was estimated by comparing the intensity of ethidium bromide staining with DNA standards or by slot blot hybridization. *F. oxysporum* has a total genome size of about 8×10^7 base pairs (bp) (16). The copy number of these plasmids was estimated to be between 25 to 50 per haploid genome by using ethidium bromide staining and approximately 10 per haploid genome by using slot blot hybridization.

Recovery of the autonomous plasmid pFOLT4. Before it was determined that pFOLT4 was linear, efforts were made to rescue the plasmid by direct transformation into *E. coli* DH5α. The plasmid pFOLT4 was separated from chromosomal DNA on an agarose gel. DNA isolated from the plasmid band transformed DH5α only at a very low frequency, typically producing only one or two ampicillin-resistant colonies per transformation experiment. All the plasmids rescued in this way appeared identical to pFT1, as ascertained by comparing restriction endonuclease patterns. After determining the linear conformation of pFOLT4 and examining its restriction endonuclease map, the rescued plasmids were presumed to have been isolated in their linear form and circularized by rare homologous recombination events upon transformation of *E. coli*. Homologous recombination between repeated sequences in pFOLT4 would have regenerated the original vector pFT1 (Fig. 1).

Recovery of the entire pFOLT4 was attempted by self-ligation of the linear plasmid with T4 ligase. Again this produced only a small number of DH5α transformants, all of which contained circular plasmids resembling pFT1. Ethidium bromide-stained gels of ligation reactions indicated that the ends of this linear plasmid were unable to be joined in

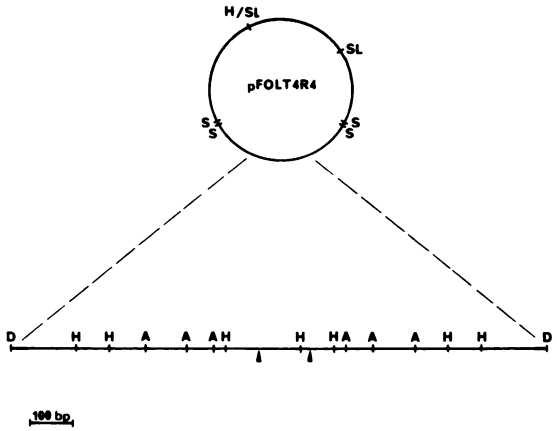

FIG. 3. Partial map of pFOLT4R4 and a 1.3-kb *Dra*I fragment containing ARS activity. The circular plasmid pFOLT4R4 derived from S1 treatment and ligation of the ends of pFOLT4 is shown with restriction enzyme sites for *Hin*dIII (H), *Sal*I (SL), and *Sst*I (S). The 1.3-kb *Dra*I fragment containing ARS activity within the 3.6-kb *Sst*I portion of pFOLT4R4 is shown below and was mapped with *Hae*III (H) and *Alu*I (A). The area between the arrowheads represents fungal DNA containing the ARS and telomere sequences shown in Fig. 7. The *Hae*III site between these arrows is formed at the presumed point of ligation of the termini of pFOLT4. Exterior to these arrows is an inverted duplication of pUC12 sequences.

this manner. Following the assumption that the ends were structurally similar to telomeres containing multiple nicks in conserved terminal sequences (3), isolated pFOLT4 was treated with S1 nuclease to cleave the DNA at any nicks. The ends were made blunt with the Klenow subunit of DNA polymerase and then joined with T4 ligase. After this treatment, a nearly full-length plasmid was recovered in DH5α in the circular conformation. The recovered plasmid was designated pFOLT4R4 (Fig. 3) and was used to characterize the unique sequences attached at termini to pFT1 sequences in pFOLT4.

Autonomous plasmids used as fungal vectors. Before pFOLT4R4 was recovered, it was determined that linear autonomous plasmids are very efficient as fungal vectors. Total DNAs isolated from transformants T4 and T9 were used to transform protoplasts. A transformation frequency of greater than 300 transformants per μg of vector was obtained when total DNA with an estimated 40 ng of autonomous plasmid was used to transform strain 73. This was over a 1,000-fold increase in efficiency compared with that with pFT1. DNAs isolated from these transformants contained the autonomous plasmid, with no incorporation into chromosomal DNAs (Fig. 4). Transformations were also performed on *F. oxysporum* f. sp. *pisi* 247 at a frequency of 2,000 transformants per μg and on a related fungus, *N. haematococca* T2, at a frequency of 120 transformants per μg of vector. DNAs isolated from these transformants also contained the full-length vector, with no evidence of integration into the high-molecular-weight chromosomal DNA (Fig. 4).

Plasmid pFOLT4R4 was used as a transformation vector and similarly gave a high frequency of transformation. *F. oxysporum* f. sp. *pisi* 247 showed the highest frequency of transformation by using our procedures. This strain consistently yielded above 3,000 transformants per μg of the vector

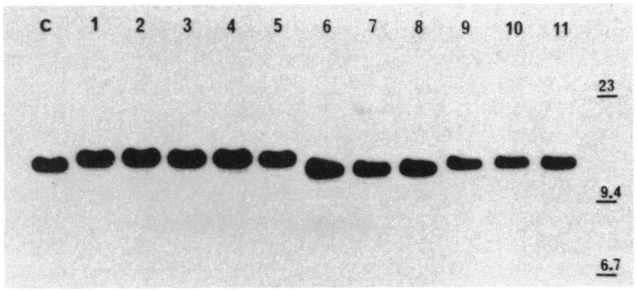

FIG. 4. DNAs from transformants produced with linear plasmids pFOLT4 and pFOLT9. Total DNAs were isolated, separated on a 0.7% agarose gel, blotted, and probed with ^{32}P-labeled pFT1. DNA from original *F. oxysporum* f. sp. *lycopersici* 73 transformant T4 was used as a control (lane c). Lanes 1 to 5, *N. haematococca* T2 transformed with pFOLT9; 6 to 8, *F. oxysporum* f. sp. *pisi* 247 transformed with pFOLT4; 9 to 11, *F. oxysporum* f. sp. *lycopersici* 73 transformed with pFOLT9. All transformants maintained the transforming plasmid in its original form autonomous from the chromosomal DNA. Numbers at the right refer to DNA size standards (kilobases).

pFOLT4R4 and consequently was used for most of the subsequent experimentation. However, other fungi such as *F. oxysporum* f. sp. *conglutinans* PHW808 and PHW722 and an unrelated fungus, *C. parasitica* EP155, also were transformed at a high rate with pFOLT4R4, and in each case the vector remained an autonomously replicating unit in the cell.

While maintained in *E. coli*, it was determined that the vector pFOLT4R4 is a supercoiled circular plasmid. To determine if there was a change in conformation of pFOLT4R4 after the transformation of fungi, DNAs were isolated from the transformants and analyzed (Fig. 5). When digested with *Sst*I, pFOLT4 (T4 DNA; Fig. 5, lane 4) and pFOLT4R4 (lane 3) showed different patterns because pFOLT4R4 was circularized by joining the doublet 1.8-kb *Sst*I fragments present at the termini of pFOLT4 (Fig. 1).

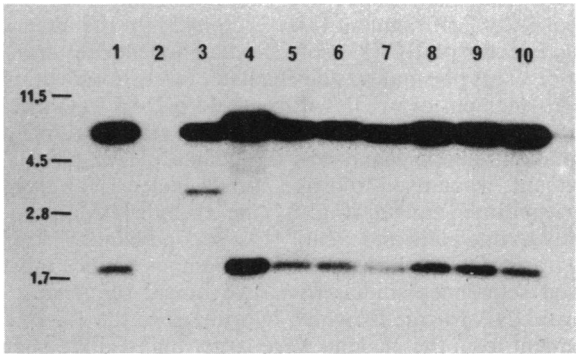

FIG. 5. DNAs from pFOLT4R4 transformants. Total DNAs were isolated from *F. oxysporum* transformants. The plasmid pFOLT4R4 was isolated from an *E. coli* clone. Samples were digested with *Sst*I, separated on a 0.7% agarose gel in TBE buffer, blotted, and probed with ^{32}P-labeled pFT1. Lanes: 2, lambda DNA digested with *Pst*I used as size markers; 3, pFOLT4R4 digested with *Sst*I; 4, DNA from the original *F. oxysporum* f. sp. *lycopersici* 73 transformant T4, which contained plasmid pFOLT4. The remaining lanes contained DNAs from pFOLT4R4 transformants: 1 and 7, *F. oxysporum* f. sp. *conglutinans* 808 transformants T1 and T2; 5 and 6, *F. oxysporum* f. sp. *conglutinans* 722 transformants T1 and T2; 8 to 10, *F. oxysporum* f. sp. *pisi* transformants T1, T2, and T3. Numbers to the left refer to DNA size standards (kilobases).

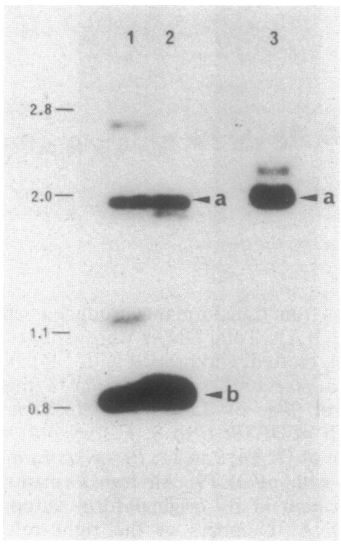

FIG. 6. Sequence similarity between cloned DNA with ARS activity and DNA from untransformed and transformed *F. oxysporum* f. sp. *lycopersici* 73. Total DNAs isolated from strain 73 transformants T9 (lane 1) and T4 (lane 2) and from untransformed strain 73 (lane 3) were digested with *Dra*I, separated on a 0.7% agarose gel in TBE buffer, blotted, and probed with the ^{32}P-labeled 1.3-kb *Dra*I fragment from pFOLT4R4 having ARS activity. Endogenous bands (a) can be seen in all three samples. The fragment (b) representing the ends of pFOLT4 and pFOLT9 can be seen only in transformants with autonomous plasmids.

Lanes 1 and 5 to 10 show DNAs from various pFOLT4R4 transformants digested with *Sst*I. Regeneration of the 1.8-kb *Sst*I fragments not present in pFOLT4R4 indicated that pFOLT4R4 was linearized when replicated in the fungus. The site of linearization apparently was the same in all the transformants tested.

Isolation and characterization of the ARS and the telomere. Fragments from pFOLT4R4 were subcloned in order to isolate the sequences necessary for autonomous replication. The 3.6-kb *Sst*I fragment (Fig. 3) cloned into the integrative fungal vector pHRC (13) conferred high-frequency transformation. This plasmid was designated pHA1. From within the 3.6-kb segment of pFOLT4R4, a 1.3-kb *Dra*I fragment was subcloned, which also conferred a high frequency of transformation. This plasmid was designated pHA2. The 1.3-kb fragment, when used to probe *Dra*I-digested DNA from the untransformed fungus (Fig. 6, lane 3), hybridized predominately to one band and weakly to a second band. Therefore, the fragment which supports autonomous replication contained sequences similar to native fungal sequences. The fungal DNA fragments which hybridized to the 1.3-kb *Dra*I fragment from the plasmid were larger than 1.3 kb, suggesting that either the *Dra*I site in the plasmid did not originate from the fungal genome DNA or some of the fungal sequences acquired by the plasmid were lost. The sequencing data described below indicate that the *Dra*I site originated in the pUC12 portion of pFT1. Figure 6 also shows DNAs isolated from strain 73 transformants T4 (lane 2) and T9 (lane 1), digested with *Dra*I and probed with the same fragment. Two major bands, identical in size to the indigenous sequence (a) and the terminal fragments of pFOLT4 and pFOLT9 (b), were found. A band the size of band b was present in all four of the following autonomous plasmids tested: pFOLT4, pFOLT9, pFOPT1, and pFOPT2 (pFOPT1 and pFOPT2 are autonomous plasmids produced in strain 247 by transformation with pFT1 [data not shown]). Since the *Dra*I site was contained in a sequence of pFT1 origin and since the four autonomous plasmids all contain *Dra*I fragments similar in size, a common sequence may exist in pFT1 into which the ARSs from the fungus are incorporated. Fine restriction mapping (Fig. 3) and subsequent sequencing of the 1.3-kb *Dra*I fragment originating from pFOLT4R4 demonstrated that it contained pUC12 sequences arranged as inverted repeats separated by a region of fungal DNA. The other lighter bands (Fig. 6, lanes 1 and 2) were fragments containing the pUC12 sequences present in both plasmids and not found in strain 73 DNA. These bands differ in pattern because of the differences in the maps of the plasmids.

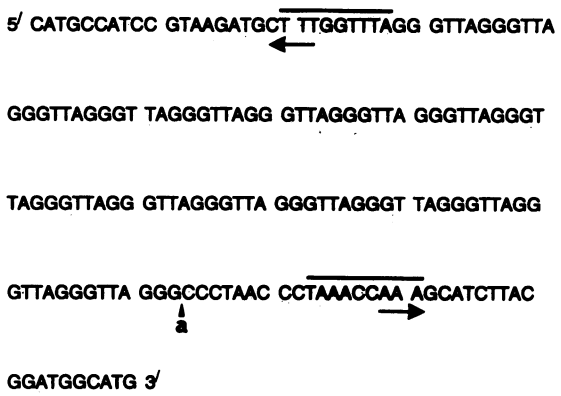

FIG. 7. Sequence derived from clones of pFOLT4R4 which shows ARS activity. Arrows point toward the pUC12 sequences that are inverted repeats. The solid lines highlight the putative ARS corresponding to the yeast autonomously replicating consensus sequence and overlapping the pUC12 sequence by 3 bp. Central are 18 repeats of the telomere sequence TTAGGG juxtaposed to 2 repeats of the complementary sequence CCCTAA. a, Presumed ligation site of termini from pFOLT4 formed during circularization of the plasmid to create pFOLT4R4 (this sequence has been given GenBank accession no. M29451).

To locate the ARSs, *Hae*III and *Alu*I subclones of the 1.3-kb *Dra*I fragment were constructed. The clones obtained were tested for transformation of *F. oxysporum* f. sp. *pisi* 247. Only fragments containing fungal DNA conferred a high efficiency of transformation. One clone was found to give greater than 3,000 transformants per μg of DNA. These subclones of the 1.3-kb *Dra*I fragment were sequenced and found to contain pUC12 sequences (Fig. 7) arranged in an inverted repeat and separated by 126 bp of DNA derived from the fungus. The fungal DNA has the telomerelike sequence TTAGGG repeated 18 times and joined to the complementary sequence CCCTAA repeated twice. Joining the pUC12 and telomeric sequences on either side were only 3 bp of unique DNA (GGT on one side and the complementary ACC on the other). The putative ligation point that created a circular plasmid from the linear pFOLT4 (Fig. 7, a) formed a unique *Apa*I restriction site. The 9-bp sequence TTTGGTTTA bridging pUC12 and telomeric regions is centered around the 3 unique bp (Fig. 7) and bears some similarity to the yeast autonomously replicating consensus sequence (A/T)TTTATPTTT(A/T) (5, 12), where P is a purine.

DISCUSSION

In vivo rearrangements resulting in autonomously replicating plasmids. Transformation of *F. oxysporum* f. sp. *lycoper-*

sici 73 with pFT1 produced autonomously replicating linear plasmids in 4 of 11 transformants tested. Three of these plasmids were studied further and were found to have arisen by rearrangement of pFT1 and acquisition of fungal DNA. Both pFOLT4 and pFOLT9 contain uninterrupted but greater-than-unit-length portions of pFT1 flanked by repeated sequences derived from fungal genomic DNA. Plasmid pFOLT7 contains rearranged portions of pFT1 flanked by fungal DNA, similar to that of pFOLT4 and pFOLT9. Examination of the pFT1 sequences contained in these plasmids suggests that pFOLT4 and pFOLT9 were produced from tandemly repeated pFT1. Typical integrative transformation of *F. oxysporum* often results in incorporation of tandemly repeated vector sequences in the chromosomal DNA, and rearrangements sometimes do occur (13). Therefore, the mechanism for production of these autonomous plasmids may have some similarities to processes involved in integrative transformation.

One of the autonomously replicating plasmids, pFOLT4, was isolated and examined in more detail. A duplicated *Dra*I fragment from either end of pFOLT4 contained ARS activity and was found to hybridize to nuclear DNA from nontransformed *F. oxysporum* 73. An examination of sequences from DNA fragments which promote autonomous replication and stabilization of the ends of the linear plasmid revealed similarities to an autonomously replicating consensus sequence and a telomere sequence. Only the telomerelike region and at most 6 additional bp of pFOLT4 display sequence similarity to strain 73 DNA, as determined by hybridizations. Sequence data revealed that the regions flanking the fungal sequences are perfect inverted repeats derived from pUC12. Since one side of pFOLT4 contains pFOXC2 sequences immediately adjacent to the terminal *Dra*I fragment (Fig. 1), it appears that the termini may have arisen by duplication of the pUC12 proximal end and translocation of these sequences to the pFOXC2 side of the molecule. The total length of the duplicated sequence is yet to be determined. An analysis of the junction region will provide more information about the mechanism by which the process occurred.

Amplification of DNA sequences in response to environmental stress has been widely reported. Gene amplification can involve production of linear or circular extrachromosomal molecules. For example, naturally occurring resistance of yeast cells to antimycin A can result from amplification of the *ADH4* locus on linear extrachromosomal elements (32). These elements are palindromic and appear to have telomeres. During transformation, the mobilization of genes for hygromycin phosphotransferase in *F. oxysporum* has parallels with this strategy of drug resistance and may occur by a common mechanism.

The precise manner by which such nonrandom rearrangements could occur is still unclear. However, any mechanism must account for (i) telomere addition to plasmid termini, (ii) creation of terminal inverted repeats, and (iii) formation of greater-than-full-length copies of the original plasmid. A possible explanation may be that by chance a sequence in the pUC12 vector is elongated by an enzyme in *F. oxysporum* with telomere terminal transferase activity. Sequences immediately 5′ to telomeres, but containing no telomere repeats, are capable of priming *Tetrahymena* telomerase (10). This hypothesis may be tested in vitro by determining if pUC12 or synthetic oligonucleotides of the sequence immediately 5′ to telomeres in pFOLT4 initiate nontemplated deoxynucleotidyl transferase activity in extracts of *F. oxysporum*.

The tandem duplication of the vector, whether initially incorporated or replicating free of the chromosome, could allow for both formation of greater-than-full-length copies of the plasmid and the potential for introducing sequence inversions (22). Tandem duplications of pFT1 may provide the opportunity for internal rearrangements resulting in the formation of terminal inverted repeats. The fate of tandemly repeated sequences during mitosis in filamentous fungi is poorly characterized. However, a few studies indicate that such duplications result in instability, possibly explained by intrastrand gene conversion or mitotic recombination. For example, the unstable mutant allele am_{126} of *N. crassa* contains a small tandem duplication, whereas a revertant obtained appears to have precisely excised this duplication (23). Likewise, a strain with a partial deletion of the tandemly repeated ribosomal DNA genes of the nucleolus organizer can amplify this sequence again within a relatively few generations, presumably by unequal sister strand recombination (25). We have noted at least one case of mitotic instability in *F. oxysporum* by introduction of tandemly repeated sequences of an integrative vector into the genome (13).

The production of these plasmids is not limited to the vector pFT1, since a similar vector, pHRC12, was rearranged in the fungus to produce an autonomous plasmid, pFOLT8. Also, *F. oxysporum* f. sp. *lycopersici* 73 is not the only fungus to produce autonomous plasmids. A related fungus, *F. oxysporum* f. sp. *pisi* 247, also produced two autonomously replicating plasmids, pFOPT1 and pFOPT2. We have yet to determine whether fungal species other than *F. oxysporum* may be able to produce similar plasmids.

ARSs and telomerelike sequences of pFOLT4. Sequences in pFOLT4 allow for stable maintenance of a linear plasmid, high-efficiency transformation, and a plasmid copy number of approximately 10 to 50 per haploid nucleus. These characteristics can be explained by the presence of functional telomere and ARS regions in the molecule. By using a procedure successful for cloning telomeres from yeast cells (27), recovery of pFOLT4 in *E. coli* was accomplished only after treating the isolated plasmid with S1 nuclease followed by DNA polymerase before ligation of the termini. This treatment cleaves the DNA at any nicks or regions of single-stranded DNA and makes undigested single-stranded regions blunt ended. The plasmid obtained in this manner, pFOLT4R4, contained a *Dra*I fragment derived from the ligated termini of pFOLT4 that could hybridize to several of the chromosomes of untransformed *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *pisi* separated by pulsefield electrophoresis (unpublished results). This fragment also hybridized to DNA from several other strains of *F. oxysporum*, the related ascomycetous fungus *N. haematococca*, and another plant pathogenic fungus, *M. grisea* (data not shown), demonstrating the conserved nature of these sequences. Figure 7 shows the cloned sequence of fungal DNA acquired by pFOLT4R4, which appears to be sufficient to support autonomous replication and to regenerate stable linear structures in the fungus. Surrounding the point marked a (Fig. 7) are repeats of the telomerelike sequence 5′-TTAGGG-3′ identical to those from telomeres of *Didymium iridis*, *Physarum polycephalum*, *Trypanosoma brucei*, and human chromosomes (2, 8, 19) and fitting the consensus sequence of a G-rich strand directed 5′ to 3′ from the interior of the molecule toward its end (1). Portions of total DNA from *F. oxysporum* with sequence similarity to this repeat are hypersensitive to Bal 31 digestion, indicating the terminal locations of the elements (unpublished results). This is

the first published sequence for a putative telomeric region from a filamentous fungus; however, an earlier report described the cloning of the terminus of chromosome VR from *N. crassa* (26). The structure of pFOLT4R4 appears to be sufficient to regenerate telomeres because after transformation of the circular plasmid into the fungal cell, the vector reverts to its linear form, pFOLT4. Presumably, the repeated sequence is recognized as a telomere by *F. oxysporum* and the introduction of nicks, normally present in the C-rich strand of telomeres, allow the circular plasmid to regenerate its linear form. To the right of position a (Fig. 7), pFOLT4R4 contains only 12 bp of the telomeric sequence, yet this apparently is sufficient to stabilize the end of the plasmid. Circular plasmids containing inverted repeats of telomeric sequences are resolved to their linear form at low frequency in yeast cells (17).

Telomeric sequences suggest the mechanism by which the circular plasmid could become linearized and maintained in the linear form but not the reason for the high copy number of pFOLT4 in the fungus. Telomeric DNAs have been used in another filamentous fungus to stabilize the linear form, but these sequences did not increase the frequency of transformation nor was the stabilized plasmid maintained at an elevated copy number (21). The increase in transformation frequency and the copy number of the plasmid are presumed to be the result of a separate ARS. Further analysis will be needed to determine the exact requirements for autonomous replication.

The plasmid pFOLT4R4 replicated as a linear autonomous plasmid in four *F. oxysporum* strains and in the other ascomycetes, *N. haematococca* and *C. parasitica*. Therefore, the putative ARS and telomere sequences in this plasmid also function in heterologous recipient strains. The ability to transform at a high frequency, the autonomous maintenance of the plasmid from the chromosome, and the ability to function in several filamentous fungi make these plasmids ideal for construction of broad-host-range fungal vectors.

Fungal vectors. Until recently, stable transformation of filamentous fungi has been possible only by integration of vectors into the chromosome of the host strain. The inefficient process of plasmid integration made transformation frequencies very low in many fungi. However, a fungal vector containing a functional ARS has been developed for the basidiomycete *U. maydis* (31). The ARS was identified from a *U. maydis* genomic library by the ability to increase the transformation frequency of an integrative plasmid. The *U. maydis* plasmid was not associated with the telomere function because fungal plasmids were circular and could be readily recovered in *E. coli*. Autonomous fungal plasmids we constructed for *F. oxysporum* are linear and associated with telomeric structures. It is unknown if ARS and telomere activity are separable or dependent upon the same sequences. There are no obvious sequence similarities between the DNAs with ARS activity from *U. maydis* and *F. oxysporum*. Since pFOLT4R4 is capable of replication in other ascomycetes, the ARS may be broadly applicable in these fungi (which include *Neurospora* spp. and *Aspergillus* spp.). The host range of the plasmid developed for the basidiomycetous fungus *U. maydis* has not been reported.

The utility of self-replicating telomeric plasmids remains to be explored. One potential application is to isolate fungal centromere sequences by selecting for genomic clones that stabilize drug resistance without selection. Artificial fungal chromosomes could thus be produced. Regardless of their potential usefulness for constructing unique vectors for fungi, further examination of ARSs and telomeric sequences on the plasmid and homologous sequences in the fungal genome will help to elucidate the mechanisms of DNA replication and chromosome maintenance in filamentous fungi.

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