Jour. Microbiol. June 1995 p. 132-135 Vol. 33, No. 2

Megabase-sized DNA Isolation and Electrophoretic Karyotype of *Fusarium oxysporum* Schlecht

Min-Seon Park and Byung-Re Min*

Department of Biology, College of Natural Sciences, Sang Myung Women's University, Seoul 110-743, Korea

(Received May 2, 1995/Accepted June 5, 1995)

To investigate the electrophoretic karyotype of *Fusarium oxysporum*, intact chromosomal DNA was separated by pulsed-field gel electrophoresis (PFGE). DNA extraction from nuclei, mycelia and protoplasts were compared with one another and with the quantity and the suitability for PFGE separation in agarose gel. As a result, the most useful extracting method for intact DNA was found to be that from protoplasts. By varying the electrophoretic conditions, 8 chromosomal DNA bands were resolved. Using the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* as size standards, the size of *Fusarium oxysporum* chromosomes was estimated to range from approximately 0.6 Mb to 6.7 Mb, and total genome size was 26.7 Mb. The suitability of electrophoretic karyotyping as a tool for strain characterization is discussed.

Key words: *Fusarium oxysporum*, PFGE, megabase-sized DNA, electrophoretic karyotype

Fusarium oxysporum belonged to imperfect fungi, is an asexual, soilborne fungus found in throughout the world and has been known to affect agricultural products under suitable environmental conditions. They produce toxic compounds which cause serious diseases in animals, plants and even humans (1, 5). Especially they affect harvesting of industrially and economically important plants. Although genetic studies of *F. oxysporum* are necessary for these reasons, they have been hindered due to some reasons: specific variations (12), existence of nuclear membrane during mitosis and imperfect sexual stage etc. Especially fungal chromosomes are, in general, too small to be accurately counted by using classical staining and light microscopic techniques. Therefore cytogenetic knowledge of fungal chromosomes is extremely limitted.

Since the introduction of pulsed-field gel electrophoresis (PFGE) in 1984 (11), rapid advance has been made even in the resolution of large DNA molecules and PFGE has become an indispensable tool for determining karyotypes of many organisms, particularly lower eukaryotes like fungi which have too small-sized chromosomes to be isolated.

However *fusaria* still have remained one of unexplored groups in this respect. The objective of this study was to isolate intact megabase-sized DNA and to define the electrophoretic karyotypes of *F. oxysporum*.

After extracting DNAs according to three different methods, the amount was compared and the optimized conditions selected for PFGE. By comparing those of *S. pombe* and *S. cerevisiae* and the chromosome numbers, each chromosome size and total genomic size was estimated.

Materials and Methods

Fungal strain and culture condition

Fusarium oxysporum Schlecht provided by Dr. Burgess of Sydney University was grown for 5 days on potato dextrose agar (PDA, Difco) at 28℃ in a rotary shaker. Conidia harvested from an agar plate was suspended in D.W. and an aliquot (1.5×10^8 conidia/ml) was transferred to 200 ml of potato dextrose broth (PDB, Difco) in a 500 ml flask. The flask was in a shaker (180 rpm) for 18 h at 28℃.

Preparation of intact megabase-sized chromosomal DNA

To produce a high-quality chromosomal DNA sample for pulsed-field gel electrophoresis, intact chromosomal DNA was prepared as follows: DNA preparation from protoplasts was executed by using the procedure of Ha

* To whom correspondence should be addressed.

et al. (4). After 3% driselase treatment for 3 hr, protoplasts were collected by centrifugation at 4,000 rpm for 15 min and resuspended in 1 M sorbitol, 0.05 M EDTA (pH 8.0) to obtain a final concentration of 1.0×10^9 protoplasts/ml. Protoplast suspension was warmed to 60℃, then mixed with an equal volume of molten 1% low gelling temperature agarose, and cast into plugs. DNA preparation from frozen mycelia without making protoplasts was performed by modification of McCluskey *et al.* (7). After filtering through Whatman No. 2, the filtrate was frozen at −70℃ in a deep freezer for 2 hr. Frozen mycelia were ground with homogenizing buffer (1 M sorbitol, 25 mM Tris-HCl, pH 8.0, 25 mM EDTA) using a grinder, and ground mycelia were resuspended in homogenizing buffer. Homogenized mycelia were pelleted by centrifugation at 6,500 rpm, resuspended in homogenizing buffer and pelleted several times as above. The final pellet was resuspended in pre-lysis buffer (0.01 M Tris-HCl, pH 7.0, 0.05 M EDTA, 0.01 M DTT) and incubated for 30 min at 30℃. After centrifugation, the precipitate was resuspended in 1 M sorbitol, 0.05 M EDTA (pH 8.0), then mixed with an equal volume of molten 1% low gelling temperature agarose, and cast into plugs. DNA preparation from mycelial nuclei was performed by using the modified method of Cohen and Stein (3). Frozen mycelia were ground with homogenizing buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 15 mM $MgCl_2$, 40 mM KCl, 25 mM ascorbic acid) using a homogenizer for 30~40 min. After centrifugation (500 rpm) for 5 min, the supernatant was recentrifuged at 1,000 rpm for 5 min. The precipitate was resuspended in resuspension buffer (0.75 M sucrose, 20 mM Tris-HCl, 0.5 mM EDTA, 15 mM $MgCl_2$). The suspension was overlayed on 2.2 M sucrose, 20 mM Tris-HCl, 0.5 mM EDTA, 15 mM $MgCl_2$, 40 mM KCl (pH 7.4) and then centrifuged at 18,000 rpm for 3 hr. The pellet was resuspended in 1 M sorbitol, 0.05 M EDTA (pH 8.0), then mixed with an equal volume of molten 1% low gelling temperature agarose, and cast into plugs.

The solidified agarose plugs prepared from three different methods were incubated at 50℃ for 48 hr in NDS buffer (0.5 M EDTA, pH 8.0, 10 mM Tris-HCl, 1% N-lauroylsarcosine) containing 1 mg/ml proteinase K. Subsequently, the plugs were washed three times with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and stored at 4℃ in 10 mM Tris-HCl (pH 8.0), 100 mM EDTA.

DNA measurement

The amount of DNA extracted was measured by using a fluorometer (Hoefer Scientific Instrument) and fluorescent dye 33258 Hoechst suggested by Cesarone *et al.* (2). Calf thymus DNA (Sigma) was used as a standard DNA.

Pulsed-field gel electrophoresis

The DNA-containing agarose plugs were cut to fit the wells and loaded. Pulsed-field gel electrophoresis was performed with a contour-clamped homogeneous electric field (CHEF) system (CHEF-DRII, Bio-Rad), using 1X TBE as a running buffer. Buffer temperature was maintained at 14℃ in all the experiments.

To obtain optimal separation, two sets of parameters were applied depending upon the sizes of the DNA molecules. Field strength, switch intervals, run time, and agarose concentrations were described in the figure legends.

After electrophoresis, the gels were stained in ethidium bromide (2 μg/ml) for 15 min, destained in TBE buffer for 40 min and visualized on a UV transilluminator. To determine the sizes of each *F. oxysporum* chromosome, well defined size standards from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Bio-Rad) were co-electrophoresed. Altogether, several agarose plugs were prepared, and electrophoretic separation was repeated to prove reproducibility.

Result analysis by image analyzer

The photographs of gels were scanned with a densitometer to measure the relative intensity of each stained band and were analyzed with a Cream-Image Analyzer.

Results and Discussion

Comparison of DNA sample preparation

DNA plugs extracted by three different methods were subjected to electrophoresis on 0.6% agarose gel and compared with one another (Fig. 1).

The amount of DNA extracted from nuclei was 24 μg/ml (of DNA sample solution), 846 μg/ml from homogenate and 96 μg/ml from protoplasts. While the homogenization method gave the highest yield, a significant amount of fragmented DNA appeared as smeared background after electrophoresis. The amount of DNA extracted from nuclei was too small to resolve banding patterns. The sample extracted from protoplast was separated as intact high-molecular weight bands without smear. According to McCluskey *et al.* (7), non-protoplasted DNA preparation was better resolved than protoplast formation in *Ustilago hordei* and *Saccharomyces cerevisiae,* but in *F. oxysporum,* we found that DNA from the protoplast formation was better for resolution.

Electrophoretic karyotype of *F. oxysporum*

Several running conditions for the CHEF gels were

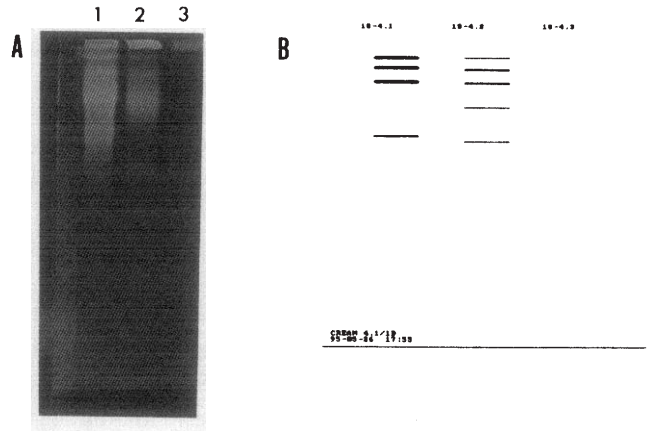

Fig. 1. Comparison of chromosomal DNA banding patterns according to the different extracting methods in *F. oxysporum*. (A) The result after 84 hr PFGE. (B) Analyzed result of (A) by image analyzer. Electrophoresis conditions: 0.6% agarose was used and run at 14℃ using 1× TBE, field inversion gel electrophoresis was carried out for 60 hr at 1.0 V/cm, 20 min forward duration and at 0.7 V/cm, 10 min reverse duration, and then followed by CHEF gel electrophoresis for 24 hr at 1.2 V/cm. Lane 1. DNA sample extracted from homogenized mycelia, 2. DNA extracted from protoplasts, 3. DNA extracted from nuclei.

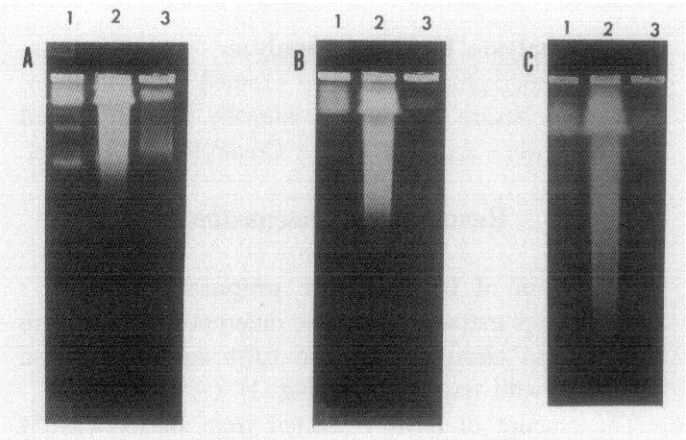

Fig. 2. Resolution of *F. oxysporum* intact chromosomal DNAs on a PFGE for 172 hr. (A) Running after 72 hr. (B) Running after 112 hr. (C) Running after 172 hr. Electrophoresis conditions: 0.6% agarose was used and run at 14℃ using 1× TBE, field inversion gel electrophoresis was carried out for 48 hr at 1.0 V/cm, 30 min forward duration and at 0.7 V/cm, 10 min reverse duration, and then followed by CHEF gel electrophoresis for 124 hr ramped 20-5 min switch interval at ramped 1.2~2.0 V/cm. Lane 1. DNA sample extracted from protoplasts, 2. DNA extracted from homogenized mycelia, 3. *S. pombe* used as a standard.

tested to resolve *F. oxysporum* chromosomes. When chromosomal DNAs were separated on 0.6% agarose gel, we found the existence of two chromosomes which were smaller than the smallest band of *S. pombe* (Fig. 2). So the two sets of parameters on which *S. cerevisiae* and *S. pombe* chromosomal bands were well separated was applied to separate *F. oxysporum* chromosomal DNA.

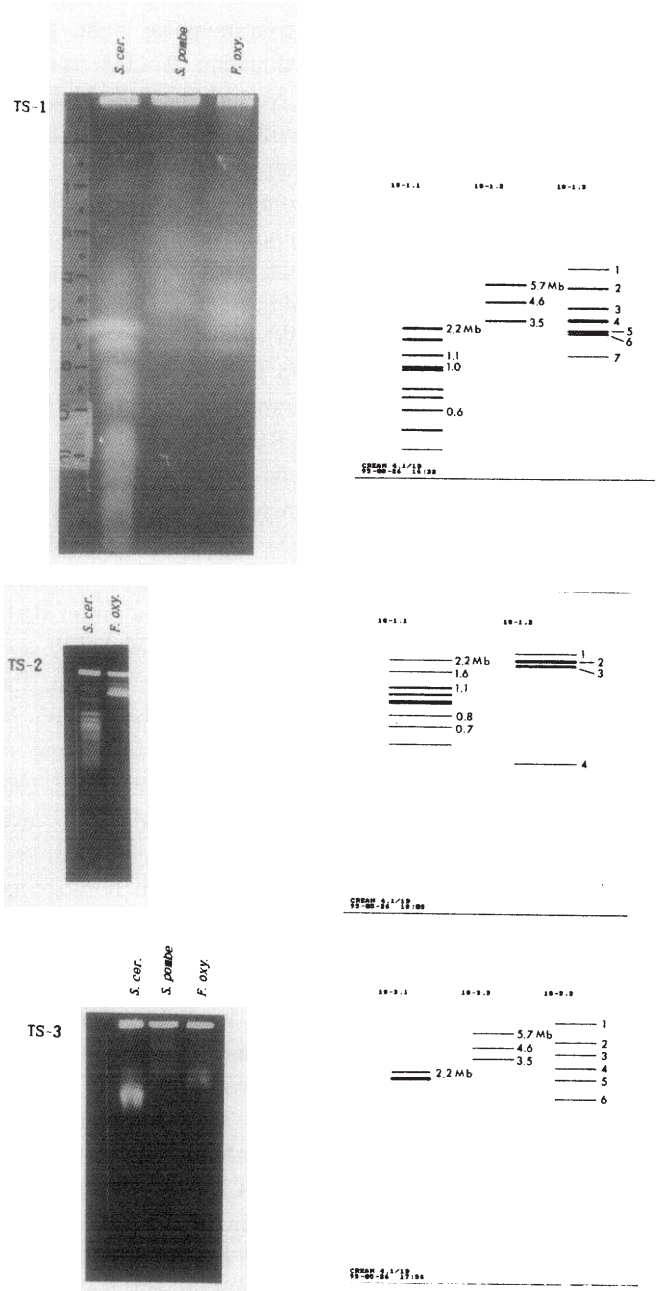

Fig. 3. CHEF gel electrophoresis of chromosomal DNA molecules of *F. oxysporum*. TS-1) Separation of DNAs ranging from 1.6 to 6.7 Mb: *S. cerevisiae*, *S. pombe* and *F. oxysporum* chromosomes were separated on 0.6% agarose gel with a reorientation angle of 106°. Conditions were 2.0 V/cm, 40 min switch interval for 45 hr, followed by 40 hr at 3.0 V/cm with a 150 sec switch interval. TS-2) Separation of the smallest chromosomal DNA of *F. oxysporum; S. cerevisiae* and *F. oxysporum* chromosomes were separated on 0.55% agarose gel. Conditions of electrophoresis: After preelectrophoresis for 1 hr at 2.0 V/cm, 1) initial 36 hr, field inversion gel electrophoresis; forward 1.0 V/cm for 30 min, reverse, 0.7 V/cm for 10 min, 2) followed by 78 hr ramped 20-2 min switch interval at ramped 1.2~2.0 V/cm. TS-3) Separation of DNAs ranging from 0.6~6.7 Mb; *S. cerevisiae*, *S. pombe* and *F. oxysporum* chromosomes were separated on 0.7% agarose. Conditions of electrophoresis: 1) initial 24 hr, field inversion gel electrophoresis; forward, 1.0 V/cm for 30 min, reverse, 0.7 V/cm for 10 min, 2) followed by 60 hr ramped 30-5 min switch interval at ramped 1.2~2.0 V/cm.

Table 1. Estimated numbers and sizes of *F. oxysporum* chromosomes separated from different conditions of PFGE

Estimated size (Mb)	TS-1	TS-2	TS-3	No. of Chromosomes
6.7	+	*	+	I
6.0	+		+	II
4.4	+		++	III
3.0	+			IV
2.5	+		++	V
1.9	+		+	VI
1.6	+		+	VII
0.6		+	+	VIII
Total genome size				26.7 Mb

TS-1: the condition for separating 2~6 Mb sized DNA, TS-2: the condition for separating <1 Mb sized DNA, TS-3: the condition for separating <1 Mb and >6 Mb sized DNA, *: multiple unresolved chromosomes, +: single chromosome, ++: doublet.

When DNA was resolved under the condition used to separate DNA pieces 2~6 Mb in size, 7 bands were consistently observed (Fig. 3, TS-1), and they ranged from approximately 1.6 Mb to 6.7 Mb. There was, however, loss of the smallest DNA of *F. oxysporum* and a few *S. cerevisiae*. To overcome this situation, we reduced the electric field to under 2.0 V/cm and separated the smallest DNA sized 0.6 Mb (Fig. 3, TS-2). After electrophoresis on 0.7% agarose gel, the smallest DNA and over 6 Mb-sized DNA were separated (Fig. 3, TS-3). Because of the relative intensity of UV light fluorescence following ethidium bromide staining, two of the 6 bands were judged to be double (Table 1).

Based on the approximate sizes previously assigned to *S. cerevisiae* and *S. pombe*, we estimated that *F. oxysporum* had 8 chromosomes sized from 0.6 Mb to 6.7 Mb, and the total genome size was 26.7 Mb.

These results agree with early cytological experiments which proposed that the *F. oxysporum* genome contains eight chromosomes (9, 10), and are in accordance with the reported karyotype of *F. oxysporum* by Kim *et al.* (6) and Migheli *et al.* (8).

In addition to providing a further molecular tool, alongside restriction fragment length polymorphisms, protein analysis of variability in this fungal genus, the electrophoretic karyotype of *F. oxysporum* is being used for the construction of chromosome-specific plasmid libraries and in gene mapping experiments.

Acknowledgement

This work was supported by a grant from Sang Myung Women's University.

References

1. **Brook, P.J. and E.P. White,** 1966. Fungus toxins affecting mammals. *Ann. Rev. Phytopathol.* **4**, 171-194.
2. **Cesarone, C., C. Bolognesi, and L. Santi,** 1979. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* **102**, 188-197.
3. **Cohen, R.J. and G.S. Stein,** 1975. Chromosomal proteins of *Phycomyces blakesleeanus*. *Exptl. Cell. Res.* **96**, 247-254.
4. **Ha, K.R., S.Y. Chang, and B.R. Min,** 1991. Interspecific protoplast fusion between *Fusarium poae* and *Fusarium sporotrichioides*. *Kor. Jour. Microbiol.* **29**, 123-129.
5. **Joffe, A.Z., J. Palti, and R. Arbel-Sherman,** 1974. *Fusarium oxysporum* Schlecht. in Iserael. *Phytoparasitica* **2**, 91-107.
6. **Kim, D.H., R.D. Martyn, and C.W. Magill,** 1993. Chromosomal polymorphism in *Fusarium oxysporum* f. sp. *niveum*. *Phytopathology* **83**, 1209-1216.
7. **McCluskey, K., B.W. Russell, and D. Mills,** 1990. Electrophoretic karyotyping without the need for generating protoplasts. *Curr. Genet.* **18**, 385-386.
8. **Migheli, Q., T. Berio, and M.L. Gullino,** 1993. Electrophoretic karyotypes of *Fusarium* spp. *Exp. Mycol.* **17**, 329-337.
9. **Min, B.R.,** 1988. Chromosomal studies on the varieties and forme specials of *Fusarium oxysporum*(I). *Kor. J. Mycol.* **16**, 157-161.
10. **Min. B.R.,** 1989. Chromosomal studies on the genus *Fusarium*. *Kor. Jour. Microbiol.* **27**, 325-330.
11. **Schwartz, D.C. and C.R. Cantor,** 1984. Separation of yeast chromosome sized DNAs by pulsed-field gradient gel electrophoresis. *Cell* **37**, 67-75.
12. **Snyder, W.C. and H.N. Hansen,** 1940. The species concept in *Fusarium* with reference to the section Martiella. *Ann. J. Bot.* **27**, 64-67.