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A Large-Insert (130 kbp) Bacterial Artificial Chromosome Library of the Rice Blast Fungus *Magnaporthe grisea:* Genome Analysis, Contig Assembly, and Gene Cloning

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Zhu, H., Choi, S., Johnston, A. K., Wing, R. A., and Dean, R. A. 1997. A large insert (130 kbp) bacterial artificial chromosome library of the rice blast fungus *Magnaporthe grisea:* Genome analysis, contig assembly, and gene cloning. *Fungal Genetics and Biology* 21, 337–347. *Magnaporthe grisea* (Hebert) Barr causes rice blast, one of the most devastating diseases of rice (*Oryza sativa*) worldwide. This fungus is an ideal organism for studying a number of aspects of plant–pathogen interactions, including infection-related morphogenesis, avirulence, and pathogen evolution. To facilitate *M. grisea* genome analysis, physical mapping, and positional cloning, we have constructed a bacterial artificial chromosome (BAC) library from the rice infecting strain 70-15. A new method was developed for separation of partially digested large-molecular-weight DNA fragments that facilitated library construction with large inserts. The library contains 9216 clones, with an average insert size of 130 kbp (>25 genome equivalents) stored in 384-well microtiter plates that can be double spotted robotically on to a single nylon membrane. Several unlinked single-copy DNA probes were used to screen 4608 clones in the library and an average of 13 (minimum of 6) overlapping BAC clones was found in each case. Hybridization of total genomic DNA to the library and analysis of individual clones indicated that ~26% of the clones contain single-copy DNA. Approximately 35% of BAC clones contained the retrotransposon MAGGY. The library was used to identify BAC clones containing a adenylate cyclase gene (*mac*1). In addition, a 550-kbp contig composed of 6 BAC clones was constructed that encompassed two adjacent RFLP markers on chromosome 2. These data show that the BAC library is suitable for genome analysis of *M. grisea.* Copies of colony hybridization membranes are available upon request.

Index Descriptors: bacterial artificial chromosome (BAC); *Magnaporthe grisea;* genome analysis; physical mapping; chromosome walk; contig.

The fungus *Magnaporthe grisea* is an important plant pathogen and is a useful model organism for studying various aspects of host–pathogen interactions. The fungus infects a wide range of grasses, including barley and millet, but it is best known as the causal agent of the blast disease of rice (*Oryza sativa*) (Ou, 1985). *M. grisea* is ideally suited for genetic and biological studies (Valent and Chumley,

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1991). The fungus is a haploid, heterothallic Ascomycete (Rossman *et al.*, 1990) and highly fertile laboratory strains have been developed (Chao and Ellingboe, 1991; Kolmer and Ellingboe, 1988; Leung *et al.*, 1988; Valent *et al.*, 1986). It has a relatively small genome size (C = 35–50 Mb) compared to other complex eukaryotes (Skinner *et al.*, 1993). A well-developed transformation system is available and numerous genes providing insight into mechanisms of fungal pathogenicity have been cloned (Kang *et al.*, 1995; Mitchell and Dean, 1995; Sweigard *et al.*, 1992; Talbot *et al.*, 1993; Xu and Hamer, 1996). Several high-density molecular linkage maps have been created containing several hundred RFLP markers and several phenotypic markers (Romao and Hamer, 1992; Skinner *et al.*, 1993; Sweigard *et al.*, 1993). Total map lengths range from 802 to over 1000 cM, yielding an average of ~47 kbp per cM. The average distance between markers is currently 282 to 348 kbp, but distances should be greatly reduced as a result of efforts to integrate several of the linkage maps (Nitta *et al.*, 1997).

The construction of overlapping large fragments of DNA (contigs) and map-based cloning are most useful for gaining insight into the biology of *M. grisea* and other eukaryotes. Large-insert libraries play a pivotal role in such research. Two of the commonly available vector systems to clone large insert DNA fragments (>100 kbp) are yeast artificial chromosomes (YAC)[2] and the newer bacterial artificial chromosomes (BAC) (Shizuya *et al.*, 1992). In the YAC cloning system, the size-selected large fragments of exogenous DNA are cloned into a linearized YAC vector and maintained in yeast as linear chromosomes. In the BAC cloning system, large fragments of DNA are cloned into an *Escherichia coli* F factor-based plasmid vector and maintained in *E. coli* as circular plasmids.

The two systems are similar in that both can easily handle large pieces (>100 kbp) of DNA, maintained at low copy numbers in the host cells (Zhang *et al.*, 1996b). However, compared to the YAC system, BAC libraries are relatively easy to construct with few chimeric clones (Ioannou *et al.*, 1994; Shizuya *et al.*, 1992; Woo *et al.*, 1994; Zhang *et al.*, 1996a). It is also easier to extract large amounts of pure BAC DNA using standard plasmid DNA isolation techniques that take advantage of the separation of supercoiled plasmid DNA from bacterial genomic DNA. Large-insert libraries offer a number of advantages over cosmid libraries. For example, if the objective is to assemble a 1-Mb overlapping contig, 49 steps would be required using a cosmid library, with the average insert size of 40 kbp, assuming 50% overlap between clones. However, if a BAC library was used (assuming a 150-kbp average size), it would require only 13 steps with a 90% probability of completing the walk (Zhang *et al.*, 1996a). BAC libraries have been constructed for a number of important plant and fungal species, including sorghum (Woo *et al.*, 1994), rice (Wang *et al.*, 1995; Zhang *et al.*, 1996a), *Arabidopsis* (Choi *et al.*, 1995; Wang *et al.*, 1996), and *Phytophthora sojae* (Arredondo *et al.*, 1997).

To facilitate genome studies, physical mapping, and map-based cloning in *M. grisea*, we report here on the construction and characterization of a high-quality BAC library from 70-15, a stable domesticated strain derived from the field isolate Guy11 (Chao and Ellingboe, 1991; Skinner *et al.*, 1993). The utility of this library is demonstrated through the isolation of *mac*1, a gene encoding adenyl cyclase, and the construction of a 550-kbp contig composed of six BAC clones that is anchored to chromosome 2 by RFLP markers. The library is further characterized for single-copy clones and clones containing repeated DNA, including those containing MAGGY, a retrotransposon. The library has been deposited at the Clemson Genome Laboratory and membranes for colony hybridization are available from Ralph A. Dean upon request. During the course of this work a ~7× genome coverage BAC library of *M. grisea* strain 4224-7-8 containing average inserts of 66 kbp was reported (Diaz-Perez *et al.*, 1996).

MATERIALS AND METHODS

Fungal Isolates and Cultural Conditions

M. grisea isolate 70-15 was kindly provided by Dr. A. Ellingboe (University of Wisconsin, Madison). Conidia were produced on V8 media (V8 juice, 40 ml, pH 7.0, per liter) as described previously (Zhu *et al.*, 1996). Medium used for growing mycelia was CM (yeast extract, 6 g; casein acid hydrolysate, 6 g; sucrose, 10 g; per liter).

Isolation of High-Molecular-Weight (HMW) DNA

HMW DNA from strain 70-15 was isolated according to Orbach *et al.* (1996). The final concentration of protoplasts imbedded in 1% low-melting-point (LMP) agarose (Bio-Rad, U.S.A.) was 1×10^9/ml.

[2] Abbreviations used: YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome; LMP, low melting point; PFGE, pulsed-field gel electrophoresis; AC, adenyl cyclase.

Preparation of BAC Vector

pBACwich was used as the cloning vector for construction of the BAC library (Fig. 1; S. Choi and Wing, unpublished data). The vector was constructed based on pBeloBAC11 (Kim *et al.,* 1996) with the addition of a 1.7-kbp fragment containing a *lox* site followed by a promoterless hygromycin-resistant gene. pBeloBAC 11, kindly provided by Drs. M. Simon and H. Shizuya, was created by introducing the *LacZ* gene into pBAC108L (Shizuya et al., 1992). pBACwich was isolated from 1 liter of overnight culture by an alkaline lysis method, as described by Zhang *et al.* (1996a). Restriction digestion and dephosphorylation of the vector were conducted as described by the above authors except that HK dephosphatase (Epicentre, U.S.A.) was used instead of CIAP. The quality of the linearized and dephosphorylated vector was tested by self-ligation and transformation into *E. coli* strain DH10B.

Preparation and Size Selection of Hin*dIII Partially Digested HMW DNA*

Partial digestion of *M. grisea* HMW DNA in gel blocks with *Hin*dIII was conducted and analyzed according to Zhang *et al.* (1996a). A preliminary experiment was performed for each HMW DNA preparation to determine the optimal conditions for partial digestion. The partial digests were separated by pulsed-field gel electrophoresis (PFGE)

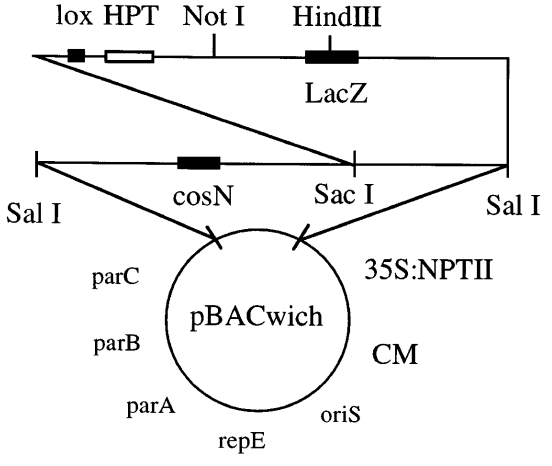

FIG. 1. pBACwich vector structure. pBACwich is derived from pBeloBAC11. CM, chloramphenicol resistance. 35S, 35S dual enhancer promoter. NPTII, kanamycin resistance. The BAC library was constructed by ligating partially digested high-molecular-weight DNA from *M. grisea* into the unique *Hin*dIII site.

in 1% LMP agarose using the CHEF DRII (Bio-Rad). Digest conditions that produced the majority of restricted DNA fragments in a size range of 100 to 400 kbp were chosen for large-scale *Hin*dIII partial digestion.

A gel containing large-scale partial *Hin*dIII digests and size markers (lambda ladder) on each side of the samples was subjected to PFGE in 0.5× TAE buffer using 5 V/cm, 10°C with a 90-s pulse for 20 h. After electrophoresis, one lane containing the lambda ladder was excised from the gel using a glass coverslip and stained with ethidium bromide to determine the size range. The gel region above 400 kbp was removed and replaced with newly prepared 1% LMP agarose and solidified at 4°C. We have found that fragments above 400 kbp are unclonable at this time. The gel was then subjected to the second size selection using the following conditions: 4 V/cm, 10°C, and a 5-s pulse for 12 h. The remaining lane with lambda ladder was excised and stained in order to identify fragments in the 100–400 kbp range. The gel region containing fragments in this size range was excised and stored at 4°C in TE.

Ligation and Transformation Conditions

The LMP agarose containing size-selected DNA was digested with Agarase (Epicentre) according to the manufacturer's instructions. A ligation mixture was prepared as described by Zhang *et al.* (1996a). One microliter of ligated DNA was transformed into 20 μl of ElectroMAX DH10B cells (BRL, USA) using a Porator (Invitrogen, U.S.A.) electroporation unit. The electroporation conditions were 13 kV/cm for field strength and 2.4 ms for time constant. After recovery in SOC medium (2% tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose, pH 7.0) for 1 h at 37°C with shaking, the cells were plated on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 12.5 μg/ml chloramphenicol, 7 μl of 200 μg/ml isopropylthio-β-D-galactoside (IPTG), and 70 μl of 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) per 100-mm-diameter plate. The recombinant clones (white) could be clearly identified after incubation at 37°C for 24 h.

Individual BAC Analysis

BAC clones were individually inoculated into 5 ml of LB broth containing 12.5 μg/ml chloramphenicol and grown at 37°C with shaking overnight. BAC DNA was extracted according to an alkaline lysis method as described by Woo *et al.* (1994). *Not*I- or *Hin*dIII-digested BAC DNA was

separated by PFGE or standard gel electrophoresis, respectively. The conditions of PFGE for the CHEF DRII (Bio-Rad) were 1% agarose gel, 0.5× TAE, 10°C, 5 V/cm, 5 s initial and 15 s final pulse time, linear ramp for 13 h.

DNA Probes

Several cosmids and plasmids from *M. grisea* libraries including cos167, cos91, cos94, A14D8, A14B10, cos247, and 4-10, which contain single-copy DNA fragments located on chromosome 2 in *M. grisea*, were used as probes (Zhu *et al.*, 1996). Numerous single-copy fragments from the ends of BACs located on chromosome 7 were chosen at random and also used as probes. AC1F2R is a 380-bp fragment amplified from the conserved region of a putative adenyl cyclase (AC) gene in strain 70-15 (W. Choi and Dean, unpublished data). Probe 23 is a 5-kbp *Eco*RI fragment isolated from a cosmid recovered from an arrayed pUI cosmid library in our laboratory (Lee and Dean, 1993). MAGGY is a ~900-bp *Hin*dIII fragment isolated from BAC clone 6G1. Radioactive labeling was performed according to the manufacturer's instructions and standard methods (Sambrook *et al.*, 1989). In all cases, the DNA used for probes was demonstrated to be single-copy by Southern analysis of genomic DNA (data not shown).

Hybridization Analysis

For Southern blotting, agarose gels were transferred onto Hybond N^+ membrane (Amersham, U.S.A.) according to the manufacturer's suggestion. Hybridization of Southern blots and colony filters was carried out in 6× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll 400, 0.02% PVP, 0.02% BSA), 0.1% SDS, and 50 m*M* phosphate buffer, pH 6.6, at 65°C. Membranes were washed down to 0.5× SSC, 0.1% SDS before being exposed to X-ray film.

Storage of BAC Clones in an Arrayed Library

Recombinant BAC colonies (white) were transferred individually by toothpick into 384-well microtiter plates (Nunc, U.S.A.) containing 50 µl FM broth (LB broth with 35 m*M* K_2HPO_4, 13.2 m*M* KH_2PO_4, 1.7 m*M* sodium citrate, 0.4 m*M* $MgSO_4$, 5.8 m*M* $(NH_4)_2SO_4$, and 4.4% glycerol). After the cells were incubated at 37°C for 24 h, several copies of the entire library were replicated into 384-well microtiter plates using a 384-pin replicating device (Nunc). Following incubation, the plates were sealed and stored at −80°C.

BAC Library Filter Preparation

The arrayed BAC library in 384-well microtiter plates was replicated onto 8× 12-cm Hybond N^+ membranes laid on LB agar containing 12.5 µg/ml chloramphenicol in a 96-well microtiter plate lid (Corning, U.S.A.) using the BioMek2000 automated workstation (Beckman, U.S.A.). Colonies from four plates were double-spotted onto each membrane. The inoculated plates were incubated at 37°C overnight. The colony filters were removed and treated according to the method of Zhang *et al.* (1996a). The filters were either used directly or stored at 4°C. Colony filters for distribution will be gridded out using a Q-bot automated workstation (Genetix, U.S.A.) located at the Clemson Genome Laboratory.

Chromosome Walking from Marker cos94

Insert fragments released by *Hin*dIII digestion of cos94 were recovered from an agarose gel and radiolabeled. After hybridization to BAC colony filters, positive clones were identified and minipreps were conducted. Probes to continue the walk were prepared by two methods. Fragments from near the ends of BACs were identified by restriction fragment analysis of positive clones, excised from gels, and radiolabeled. Probes from the ends of BAC clones were also prepared by unidirectional PCR using primers in the pBACwich vector immediately flanking the insert. The two primers used were BACL (5′-TCGACCTGCAGGCATGC-3′) and BACR (5′-GACACTATAGAATACTCAAG-3′). Unidirectional PCR was performed in 500-µl microcentrifuge tubes (U.S.A./Scientific Plastics) in a 50-µl volume containing reaction buffer (Epicenter), 100 µ*M* each nucleotide (except dCTP; 1 µ*M*), 20 µCi [α-^{32}P]dCTP (3000 Ci/mmol), 2.5 µ*M* Mg^{2+}, 2 µ*M* primer BACL or BACR, 100 ng BAC DNA, and 1 unit *Tfl* polymerase (Epicenter). Following 5 min at 94°C, 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 5 min were performed in a Programmable Thermal Blok II (Lab Line). Reaction products were purified by Sephedex G-50 (Sigma, U.S.A.) chromatography and used as probes. To confirm each walk step, DNA from positive hybridization clones was ex-

tracted and subjected to Southern hybridization analysis. Each clone shared several hybridization bands with the previous walk step clone.

RESULTS

Construction of the M. grisea *BAC Library*

A *M. grisea* BAC library containing 9216 clones was constructed from the rice infecting strain 70-15 in a novel BAC vector (pBACwich). This vector may be useful for direct selection of transformants when introduced into *M. grisea* as described under Discussion. The library was constructed from *Hin*dIII partially digested fragments of DNA from strain 70-15 that were size-selected twice by PFGE. The method of double size selection in a single gel used in this study greatly reduced the amount of DNA that is usually lost when HMW DNA is size selected on two separate gels. Approximately 25 ng of the size-selected DNA was ligated to 25 ng of vector (~10–20 molar excess). One microliter of the 100-μl ligation mixture, when transformed into *E. coli* competent cells (DH10B), yielded approximately 240 recombinant clones—representing 56% of the total transformants. Therefore, 1 ng of ligated size-selected DNA produced 960 recombinants for a transformation efficiency of almost 10^6 CFU/μg. For small pUC control plasmids, efficiencies $>1 \times 10^{10}$ CFU/μg were routinely observed.

Insert Size Distribution of the Clones in the BAC Library

To evaluate the suitability of the *M. grisea* BAC library for physical mapping, genome analysis, map-based cloning, and construction of overlapping contigs, the BAC library was examined for insert size distribution, clone representation, and the proportion of clones containing single-copy sequences. Insert size was carefully determined by restriction enzyme analysis of 137 BAC DNA minipreparations from random clones in the library. As shown in Figs. 2A and 2B, the digested BAC DNA was separated by either standard agarose gel electrophoresis for *Hin*dIII digestion or on a CHEF gel for *Not*I digestion. The insert size of each clone was determined by adding up the sizes of all *Not*I fragments then subtracting the size of the vector (11 kbp). The distribution of insert size is shown in Fig. 3 by plotting groups of clones in the same size category versus the frequency of each group. The *M. grisea* BAC library has an average insert size of 130 kbp and contains 9216 individual clones, which represents in excess of 25 genome equivalents based on the assumption that the genome size of strain 70-15 is 40 Mbp. The insert size of the 137 clones used to evaluate this library ranged from 75 to 200 kbp; over 75% of the tested clones had inserts larger than 120 kbp (Fig. 3). We have only identified two BAC clones ($<0.5\%$) from extensive analysis of >500 clones that appear empty or contain an insert <75 kbp. Theoretically, the probability of finding a particular clone in this library is greater than 99.9%.

To confirm that the insert DNA fragments in this BAC library were from *M. grisea* DNA, the BAC DNA on the gels shown in Figs. 2A and 2B was transferred to Hybond N^+ membranes and hybridized to the total genomic DNA from strain 70-15 (Figs. 2C and 2D). Many BAC clones gave strong hybridization signals. After extended exposure to X-ray film, all DNA bands (except vector) in Figs. 2A and 2B were observed. The results indicate that a significant portion of the BAC clones contain repeated DNA sequences. The weak signals, on the other hand, suggest single-copy DNA.

Proportion of BAC Clones Containing Repetitive Sequences

As shown in Figs. 2C and 2D, many BAC clones contain highly repetitive DNA sequences. To further investigate the proportion of BAC clones containing repeated sequences in this library, total genomic DNA from strain 70-15 was hybridized to filters harboring 4608 clones (Fig. 4). According to the intensity of hybridization signals, two categories were scored: putative single-copy- and repetitive-sequence-containing BAC clones. The putative single-copy BAC clones showed very weak or no hybridization signals, in contrast to clones designated as those containing repetitive sequences. In summary, ~53% of the total 4608 BAC clones were tentatively identified as containing exclusively single-copy DNA fragments, while 47% contained repetitive sequences.

To further evaluate whether clones which hybridized very weakly contained single-copy DNA, 20 clones assigned as single-copy and 20 clones assigned as containing repetitive DNA were randomly chosen, and DNA was isolated, restriction digested with *Hin*dIII, and analyzed by Southern blotting using ^{32}P-labeled total genomic 70-15

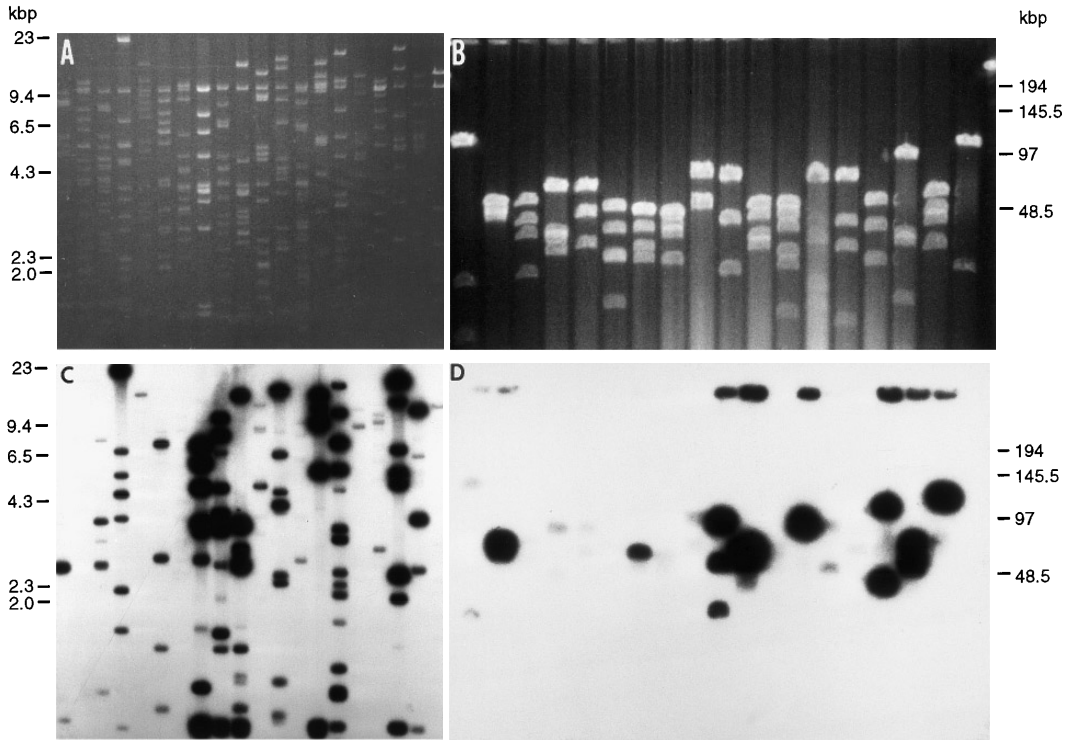

FIG. 2. Analysis of random BAC clones from the *M. grisea* BAC library. (A) *Hin*dIII-digested BAC clones separated by standard gel electrophoresis. Note the 11-kbp vector band in all lanes. (B) *Not*I-digested BAC clones analyzed by pulsed-field gel electrophoresis (PFGE). (C,D) The gels from A and B were blotted and hybridized with total genomic DNA from 70-15. Certain bands hybridized strongly, indicating highly repetitive DNA, whereas others could be visualized only following longer exposures, indicating single- or very-low-copy DNA sequences. The vector band did not hybridize.

DNA as a probe (Fig. 5). Nine of 20 (45%) clones classified as single-copy and 1 of 20 (5%) classified as containing repetitive DNA were estimated to lack repetitive DNA. Repetitive DNA was deemed present if hybridization bands of differing intensity could be observed in a particular clone. All DNA fragments showed hybridization signals after several days exposure to X-ray film with the exception of vector band (data not shown). Thus, the proportion of clones being exclusively single-copy DNA was corrected to ~26% (45% of 53% plus 5% of 47%). It was also noted that hybridization bands at ~900 bp were shared by many clones (16 of 20 clones; 80%) containing repetitive DNA. The bands were cloned and shown by DNA sequencing to be MAGGY, a retrotransposon previously characterized by Farman *et al.* (1996). Therefore, ~38% (80% of 47%) of BAC clones in our library contain MAGGY. A similar estimate (~34%) for the percentage of BAC clones containing MAGGY was determined by library colony hybridization (data not shown).

Representation of Genome Coverage in the BAC Library and Recovery of an Adenyl Cyclase Gene

According to theory, the probability of finding a particular clone in the BAC library is greater than 99.9%. This high probability is very important for all aspects of genome analysis. To test the coverage of the library, unlinked single-copy DNA fragments from BACs located on chromosome 7 and a cluster of single-copy markers from chromosome 2 (as described under Materials and Methods) were used to screen 4608 clones (>12× genome coverage). A minimum of 6 clones was found in each case, with an average of 13 ± 7. For example, the filters were probed with a 380-bp PCR product AC1F2R. AC1F2R was amplified from the conserved region of the adenyl cyclase gene in *M. grisea* by a pair of degenerate primers and confirmed as a single-copy gene by Southern hybridization (W. Choi and Dean, manuscript in preparation). The probe

hybridized to 6 clones. *Hin*dIII digestion patterns of BAC DNA isolated from these clones revealed that they shared at least 45 kbp (Fig. 6). To confirm that these clones contained the genomic region corresponding to the AC1F2R fragment, the gel was blotted and hybridized with AC1F2R. All 6 clones displayed a strong hybridization signal to a 2.6-kbp *Hin*dIII fragment (data not shown). These BAC clones were shown to contain a functional adenyl cyclase gene (*mac*1; W. Choi and Dean, manuscript in preparation).

Production of a 550-kbp Contig from Chromosome 2 Using the BAC Library

To further demonstrate the usefulness of this library we performed a chromosome walk from the RFLP marker cos94 located on chromosome 2 (Sweigard *et al.,* 1993). As shown in Fig. 7, a region of about 550 kbp was covered in only five walk steps using our BAC library. The direction of the walk from cos94 was determined by hybridization of flanking markers. In this example we identified the RFLP marker cos167, located 0.5 cM from cos94, after two walk steps (Zhu *et al.,* 1996).

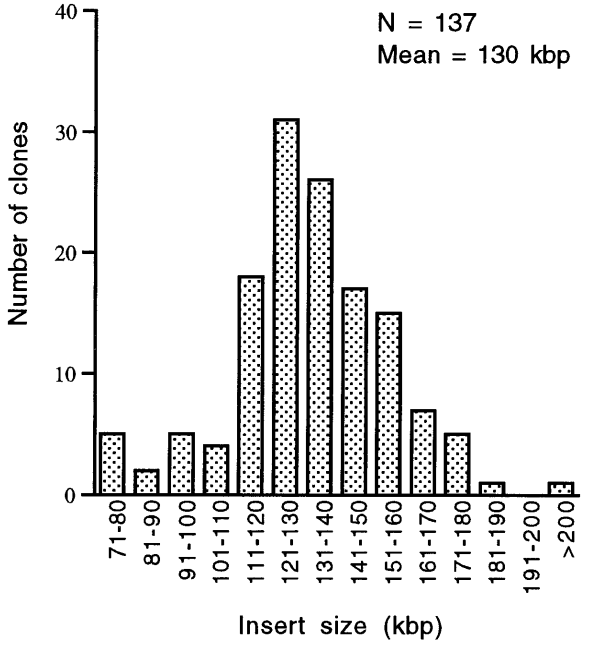

FIG. 3. Insert size distribution of BAC clones in the *M. grisea* BAC library. To estimate the insert size range, BAC DNA from 137 randomly chosen clones was analyzed, as shown in Fig. 2B. The results demonstrated that the average insert size is 130 kbp, with over 75% of the clones having insert sizes >120 kbp.

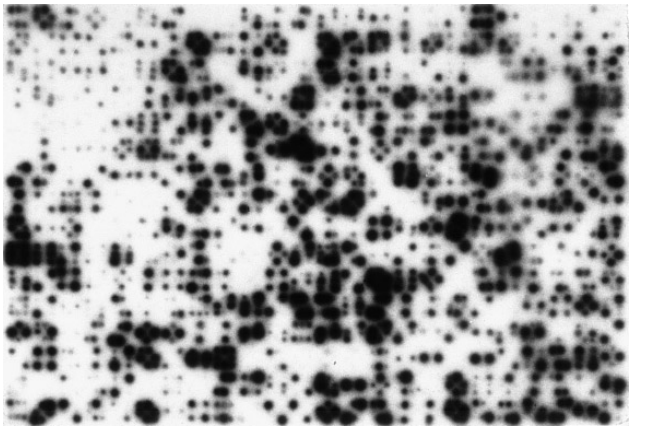

FIG. 4. Determination of BAC clones containing repeated sequences. The percentage of BAC clones containing repetitive sequences in the library was estimated following hybridization of total genomic DNA from strain 70-15 to filters harboring 4608 colonies. The three filters used for this analysis were scored as described in the text and one of them is shown here. The results indicated that ~47% contain highly and moderate repetitive sequences, while ~53% putatively contain single- and low-copy DNA.

DISCUSSION

Large-insert DNA libraries are essential for genome analysis, physical mapping, chromosome walking, map-based cloning, and whole genome sequencing. Critical components of such libraries include a large insert size, low levels of chimeric clones, genome representation, and clone stability (Woo *et al.,* 1994; Zhang *et al.,* 1996a). BAC libraries meet these criteria and therefore are most suitable for genome research (Choi *et al.,* 1995; Ioannou *et al.,* 1994; Shizuya *et al.,* 1992; Woo *et al.,* 1994; Zhang *et al.,* 1996a,b).

The average insert size of this BAC library is 130 kbp, which is comparable to the majority of human and plant BAC libraries constructed to date (Cai *et al.,* 1995; Choi *et al.,* 1995; Ioannou *et al.,* 1994; Shizuya *et al.,* 1992; Wang *et al.,* 1996; Woo *et al.,* 1994; Zhang *et al.,* 1996a). Assuming that the genome size of strain 70-15 is 40 Mbp, the library represents >25 genome equivalents. This statistically means there is a >99.99% chance of finding at least one specific BAC clone when screening with a specific sequence.

Although the insert size of our BAC library is severalfold smaller than that of YAC libraries, it is easier to handle and any deficiencies can be compensated for by the construction of several BAC libraries using different restriction enzymes. Additional BAC libraries are presently being constructed.

A most recent report by Diaz-Perez *et al.* (1996) describes the construction of a BAC library for strain 4224-7-8 of *M. grisea.* A BAC library has also been constructed for *Phytophthora sojae* (Arredondo *et al.*, 1997). In both cases, however, the average insert size is relatively small (66 kbp for *M. grisea* and 55 kbp for *P. sojae*). The reasons for this are unclear, although based on our experience it does not appear related to clone toxicity resulting from fungal gene expression in *E. coli.* The problem may involve the isolation of sufficient size-selected DNA following separation and purification on two gels. Single size-selected DNA typically yields clones with small inserts. We found it a great advantage to perform double size selection in the same pulsed-field gel in order to obtain an adequate quantity of large DNA fragments for library construction.

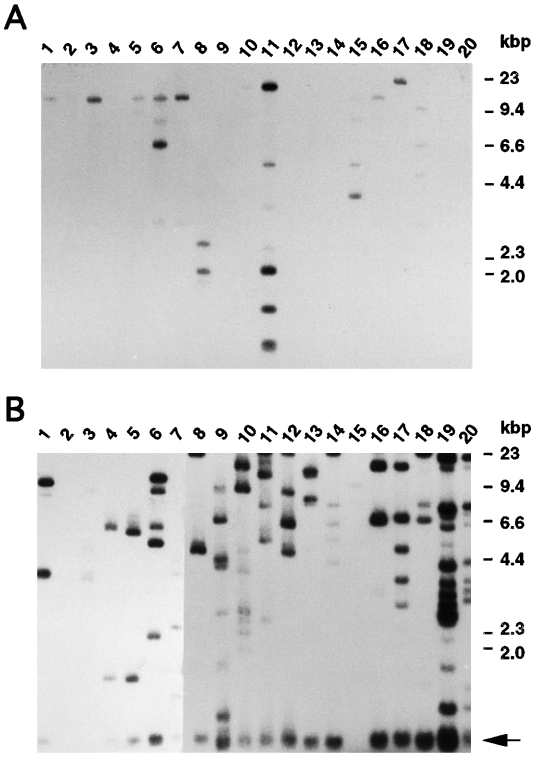

FIG. 5. Southern blot analysis of BAC clones likely containing single- or low-copy (A) and repetitive DNA (B). Approximately 1 μg of DNA from selected clones was digested with *Hin*dIII, size-separated on an agarose gel, transferred to a nylon membrane, and probed with radiolabeled total genomic DNA. In A, the presence of one or more hybridization bands in lanes 1, 3, 5–8, 11, and 15–18 indicates these clones contain repetitive DNA. In B, hybridization bands were detected in all lanes, except for lane 2, indicating the presence of repetitive DNA. Arrow indicates hybridization bands corresponding to MAGGY.

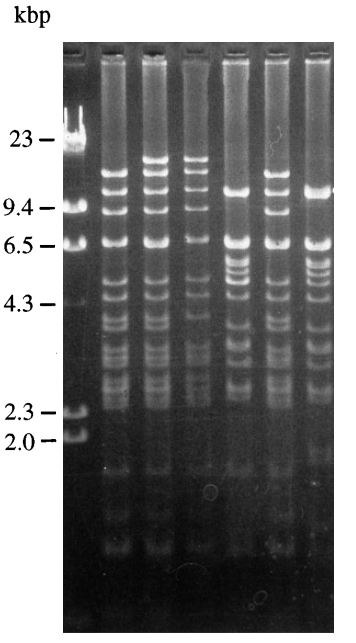

FIG. 6. Analysis of BAC clone identified using AC1F2R as a hybridization probe. DNA from six BAC clones that hybridized to radiolabeled AC1F2R was digested with *Hin*dIII and fractionated by standard agarose gel electrophoresis. The digestion patterns clearly show that these clones share several similar size fragments, suggesting considerable overlap. Southern analysis showed that AC1F2R recognized a 2.6-kbp *Hin*dIII fragment in all six clones.

The BAC library constructed in strain 70-15 should be useful to *Magnaporthe* researchers for several reasons. The strain used for library construction is a fully domesticated stable isolate that infects rice (Chao *et al.*, 1991). It is also closely related to Guy11, a wild isolate for which RFLP maps are available (Skinner *et al.*, 1993; Nitta *et al.*, 1997). We have previously demonstrated that RFLP markers derived from Guy11 can be used to readily map genes in strain 70-15. RFLP markers from a cross between Guy11 and strain 2539 were used to rapidly and efficiently fine map *app*1, a gene required for appressorium formation, to a central region of chromosome 2 (Zhu *et al.*, 1996). The BAC library will be useful not only in isolating specific genes by map-based cloning, but also in future work on integrating genetic and physical maps.

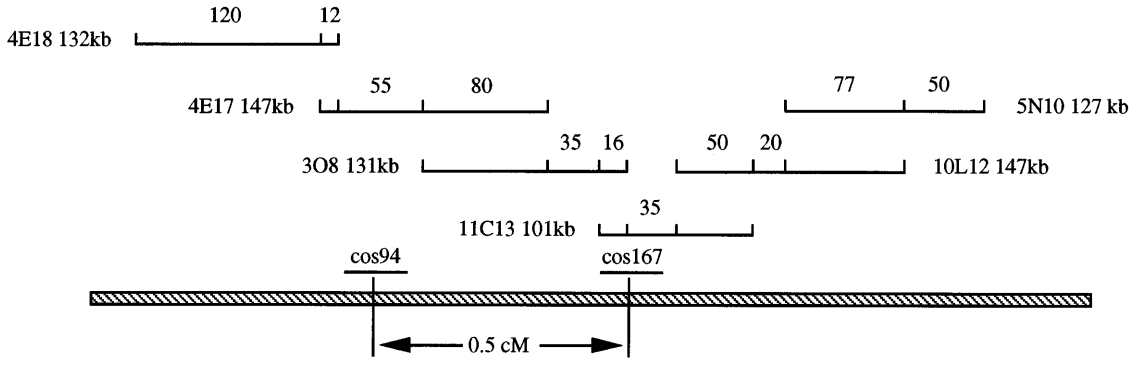

FIG. 7. A 550-kbp contig of BAC clones from chromosome 2. The *M. grisea* BAC library was used to initiate a chromosome walk from RFLP marker cos94. Ends of the BAC clones were used as hybridization probes. After two steps of walking, clone 11C13 was found to cover marker cos167, located 0.5 cM away from cos94. Numbers on each BAC clone represent the length of the shared or unique fragments in kbp.

To determine clone representation of our BAC library, unlinked single-copy probes were used to screen 4608 BAC colonies (>12× genome coverage). A minimum of 6 BAC clones were identified in each case, with an average of 13—close to the expected number. The 6 BACs containing a *M. grisea* adenyl cyclase (*mac*1) gene identified by probe AC1F2R (Fig. 6) share many bands indicating that the BAC clones overlap well. It would thus appear that this library will be most useful for genomic analysis of *M. grisea.* Although this study did not test the stability of *M. grisea* BAC clones or the presentation of chimeric clones, data from other researchers have shown that DNA inserts larger than 300 kbp can be stably maintained over 100 generations without detectable rearrangements, and few, if any, chimeric clones have been detected by fluorescent *in situ* hybridization (Jiang *et al.*, 1995; Shizuya *et al.*, 1992; Woo *et al.*, 1994). The use of commercially available DH10B cells (BRL, U.S.A.) as host may partially account for this stability (Hanahan, 1983). DH10B contains many mutations needed for large DNA fragment cloning, such as recombination deficiency (*recA*) and the cell wall defect shared with its ancestral strain, MC1061 (Hanahan, 1983). The latter facilitates high-efficiency electroporation.

The utility of our BAC library was further demonstrated by the construction of a 550-kbp contig composed of only six BAC clones. This contig spanned two closely linked RFLP markers, separated by 0.5 cM on chromosome 2. From Fig. 7, the ratio between physical distance and genetic distance for this region can be estimated at ~300 kbp/cM, which is much greater than the average ratio for *M. grisea* (~47 kbp/cM). Several other RFLP markers that cosegregate with cos167 were not covered in our 550-kbp contig, providing further evidence that this region of chromosome 2 is suppressed for genetic recombination. Additional work is required to elucidate the cause of suppressed recombination in this region. One possibility is that it may contain a centromere.

For construction of a physical map and overlapping contigs of a large chromosome area, it is essential to identify single-copy BAC clones in the library. A contig map from *Aspergillus nidulans* has been made using a random cost approach from a cosmid library (Wang *et al.*, 1994; Xiong *et al.*, 1996). By probing the cosmid library without replacement using single-copy clones, a binary clone/probe hybridization matrix was generated, and thus each clone was assigned a particular digital "call number." Clones were then placed into their inferred order along the chromosome by an efficient algorithm and visualized as a physical map (Wang *et al.*, 1994; Xiong *et al.*, 1996). From our total genomic DNA hybridization data, we show that ~26% of the BAC clones most probably contain single-copy DNA fragments exclusively. This observation will serve as a good starting point toward *M. grisea* genomics.

Recent advances in DNA sequencing technologies have demonstrated that whole genomes can be sequenced accurately and efficiently. BAC libraries are ideally suited for whole genome sequencing projects because of their large insert size and ease of DNA isolation that lends itself to automation. Whole genome sequencing using BACs as templates is being used for sequencing the human and *Arabidopsis* genomes (Venter *et al.*, 1996). As the cost of genome sequencing decreases it will be possible and practical to sequence the genomes of many other important species. *M. grisea* as an important fungal pathogen of rice is an ideal candidate. With the availability of this new BAC library and the Diaz-Perez *et al.* (1996) library and a

coordinated effort within the *Magnaporthe* genome community, it is now time for discussions concerning sequencing large regions of the *M. grisea* genome with the eventual goal of sequencing the entire genome. The outcome of such an effort would produce valuable information in the areas of fungal biology and plant pathology.

The novel BAC vector used in this study (pBACwich) contains several important features (Fig. 1; S. Choi and Wing, unpublished data). In addition to the *lacZ* gene, which allows blue and white screening for recombinants, the vector contains a *lox* site followed by a promoterless hygromycin-resistant gene (Qin *et al.*, 1994). It has been demonstrated that the Cre–*lox* site-specific recombination system works efficiently in mammalian cells (Fukushige and Sauer, 1992; Sauer, 1993), plants (Bayley *et al.*, 1992), and fungi (Sauer and Henderson, 1990). A number of appropriate recipient strains have been created recently by transformation with a plasmid containing the *A. nidulans gpd*A promoter fused to a *lox::hph* cassette using the *bar* gene as a selectable marker. In the future, we are planning to transform these recipient strains with BAC clones while selecting for hygromycin resistance to test the efficiency of the Cre–*lox* recombination in *M. grisea.* Direct BAC transformation of large DNA inserts will greatly facilitate map-based cloning of simple inherited loci, clustered loci, and quantitative traits and may reduce potential position effects associated with random integration throughout the genome (Mann *et al.*, 1988; Miller *et al.*, 1987; Zhang *et al.*, 1996b).

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