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SHORT COMMUNICATION

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Construction of a bacterial artificial chromosome (BAC) library of *Coprinus cinereus*

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Abstract A bacterial artificial chromosome (BAC) library of the genomic DNA of *Coprinus cinereus* strain MP#2 was constructed using the BAC vector pBACTZ, which carries the *C. cinereus trp1* gene. The library consists of 1536 clones. Analysis of inserts in some of the clones suggested that the library covers five times the *C. cinereus* genome. Screening of the BAC clones using ten markers mapped on nine different chromosomes also indicated that the library is likely to cover the whole length of the genomic DNA. We show an example of transformation of *C. cinereus* with BACs containing inserts of longer than 170 kb.

Key words Bacterial artificial chromosome (BAC) · Complementation · *Coprinus cinereus* · Library construction · Transformation

Introduction

Coprinus cinereus is a model organism in the basidiomycetes, providing a useful system for studies of fungal development (Moore 1998; Kües 2000; Kamada 2002). The nuclear genome of *C. cinereus* is composed of 37.5 megabases (Mb) (Dutta 1974) and separated into 13 chromosomes ranging in size from approximately 1 to 5 Mb (Pukkila and Lu 1985; Pukkila 1992). A linkage map, based on random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers, has been constructed, which comprises 13 linkage groups corresponding to the 13 chromosomes (Muraguchi et al. 2003). Since the linkage map was constructed using the monokaryotic fruiting strain #326 as a parent, mutations induced in #326, including those affecting dikaryotic phenotypes, such as mating reactions, fruit body development, and meiosis, can easily be mapped to particular chromosomes by screening RAPD markers from the linkage map for those that cosegregate with their phenotypes.

To facilitate map-based cloning of genes responsible for mutations in this fungus, we constructed a bacterial artificial chromosome (BAC) library of the genomic DNA of *Coprinus cinereus* strain MP#2 using a derivative of BAC vector pBeloBAC11 (Kim et al. 1996), which carries the *C. cinereus trp1* gene (Skrzynia et al. 1989) as a selectable marker. The BACs can be used to transform a *trp1*$^-$ auxotroph of *C. cinereus*. Because BACs carry large inserts of longer than 100 kb, the library covers the whole genome with a relatively small number of clones, and hence we should be able to identify a clone that complements a mutation by a small number of transformation experiments using the BACs.

Materials and methods

Coprinus cinereus strain MP#2 (*A91B91*) is a member of a mapping population used to construct a linkage map (Muraguchi et al. 2003). *C. cinereus* strain #292 (*A3B1 trp1-1,1–6*) was obtained from Dr. M.E. Zolan. A standard homokaryotic strain, #5302 (*A2B2*), is a stock culture in our laboratory. MYG medium (1% malt extract, Difco; 0.4% yeast extract, Difco; 0.4% glucose, 1.5% agar, Rao and Niederpruem 1969) and MYG medium supplemented with 0.01% (w/v) tryptophan were used for mycelial cultures of the strains not requiring tryptophan and strain #292 requiring tryptophan, respectively.

To insert the *trp1* gene (3 kb) (Skrzynia et al. 1989) into one of the four *Sac*I sites of the vector pBeloBAC11 (Kim et al. 1996), the *trp1* gene with the *Pst*I linker (the generous gift of M.E. Zolan) was modified to carry the *Sac*I sequence at both ends. The vector was partially digested with *Sac*I

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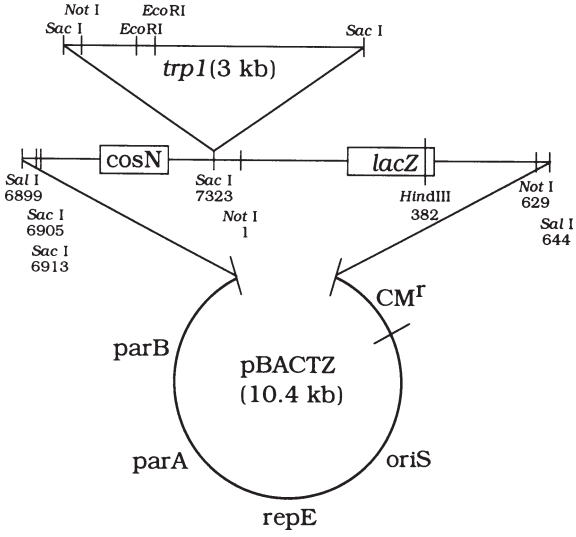

Fig. 1. pBACTZ vector structure. pBACTZ is derived from pBeloBAC11. *CM*^{r} is the gene for chloramphenicol resistance in *Escherichia coli*. The *trp1* gene of *Coprinus cinereus* was introduced into a *Sac*I site (position 7323) of pBeloBAC11. The *numbers* in the figure indicate the positions of the respective restriction sites. The BAC library was constructed by ligating partially digested high molecular weight DNA from strain MP#2 of *C. cinereus* into the unique *Hin*dIII site of pBACTZ

and fractionated in a 0.8% agarose gel. The 7.4-kb single-cut product was excised from the gel, purified, dephosphorylated with calf intestine alkaline phosphatase (CIAP), and ligated with the *trp1* gene. The ligation product was introduced into *E. coli* DH10B (Invitrogen, Carlsbad, CA, USA), and transformants were selected on LB (Luria-Bertani broth) plates containing chloramphenicol (CM) (12.5 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), and isopropyl-1-thio-β-D-galactoside (IPTG). Several plasmids from blue colonies were subjected to miniprep followed by digestion with *Eco*RI, which indicated that the *trp1* gene was integrated into the *Sac*I site at the position 7323 of pBeloBAC11 in one of the plasmids, named pBACTZ (Fig. 1).

To prepare pBACTZ efficiently, pBACTZ was fused with a high-copy plasmid pUC118, as described by Luo et al. (2001). Both pUC118 and pBACTZ were digested with *Hin*dIII. The linearized pUC118 was dephosphorylated and ligated with the linearized pBACTZ. The ligated DNA was used to transform DH10B-competent cells. Transformants were selected on LB plates containing 50 μg/ml ampicillin, 12.5 μg/ml CM, X-Gal, and IPTG. The plasmid constructed, named pUC-BACTZ, is a bireplicon plasmid and exists in a high copy number in *E. coli*.

The pUC-BACTZ plasmid DNA was isolated by a plasmid isolator (Kurabo Model PI-50). The plasmid DNA was digested with *Hin*dIII at 37°C for 3 h, followed by treatment by CIAP at 37°C for 1 h. The sample was then separated on 0.8% agarose gel. A band of pBACTZ at 10.3 kb was cut out from the gel and passed through a pinhole bored at the bottom of a microtube by centrifugation, extracted with an equal volume of phenol, and frozen at −20°C for 30 min. The emulsion containing pieces of the crushed gel was centrifuged for 10 min at 4°C. The upper phase was extracted twice with chloroform/isoamyl alcohol (24:1, v/v). The DNA sample was ethanol-precipitated, rinsed with 70% ethanol, air-dried, and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Protoplasts from oidia of strain MP#2 were prepared by the method of Zolan et al. (1992) with partial modifications mentioned below. Agarose plugs containing 3×10^8 protoplasts/ml were subjected to partial digestion with *Hin*dIII, and the partially digested DNA was fractionated by two-phase electrophoresis as described by Nakamura et al. (1997). Two fractions of DNA (100–130 kb and 160–300 kb) were excised from the unstained gel using the EtBr-stained outer edge of the lane as a guide. The high molecular weight DNA was electroeluted from the gel slices as described by Strong et al. (1997). After the electroelution, DNA was drop-dialyzed against 0.5× TE on 0.025-μm type VS filter membrane (Millipore, Bedford, MA, USA) in a Petri dish set on ice for 2 h. If DNA concentration was low, the DNA was concentrated by drop-dialysis against 0.5× TE with 20% polyethylene glycol (PEG 8000; Sigma-Aldrich, St. Louis, MO, USA) as described by Lijavetzky et al. (1999).

About 25 ng of the size-fractionated DNA was ligated to 10 ng *Hin*dIII digested pBACTZ in a final volume of 50 μl as described by Wing et al. (1995) except that the ligation was performed at 4°C overnight. Next, the reaction mixtures were desalted by drop-dialysis against 0.5× TE for 2 h on ice, and then mixed with an equal volume of 2 mM polyamines (100 mM stock: 75 mM spermidine-3HCl, 30 mM spermine-4HCl). Two microliters of the mixed samples were used to electroporate 20 μl DH10B cells in a 0.1-cm chamber using a Gene Pulser II (Bio-Rad, Hercules, CA, USA) at 100 ohms, 1.3 kV, and 25 μF. Transformed cells were selected on LB plates containing CM (12.5 μg/ml), X-Gal, and IPTG. White recombinant BAC colonies were transferred individually into 96-well microtiter plates (Nunc, Roskilde, Denmark), incubated at 37°C for 18 h, and stored at −80°C as described by Nakamura et al. (1997).

BACs, containing *C. cinereus* DNA, were isolated from 5 ml of overnight cultures by the alkaline lysis method as described by Wing et al. (1995) with the modification of treating DNA solutions with RNase A (final concentration, 0.1 mg/ml) and phenol/chloroform/isoamyl alcohol (25:24:1, v/v). The BAC DNA was resuspended in 50 μl TE. Typically, 10 μl of the DNA solution was used for restriction digestion analysis and for transformation of 100 μl protoplasts at a concentration of 10^9/ml.

Membranes for colony hybridization were prepared by inoculating 192 clones from two 96-well microtiter plates on 12 × 8 cm Hybond-N+ filters (Amersham Biosciences, Little Chalfont, UK) laid on LB agar containing 12.5 μg/ml CM, and incubated at 37°C overnight. The membranes were treated according to the standard method for colony hybridization (Sambrook and Russell 2001). The membranes were hybridized using Gene Images Random-Prime Labeling and Detection System (Amersham Biosciences).

Protoplasts were prepared from oidia of #292 as described by Zolan et al. (1992) with the modification of using

50 mg/ml of Lysing Enzyme, L1412 (Sigma-Aldrich), instead of Novozyme 234. Transformation of protoplasts was performed by the Ca^{2+}-PEG method as described by Binninger et al. (1987): 100 µl protoplasts at a concentration of 10^9/ml was treated with 10 µl BAC DNAs isolated by the alkaline lysis method.

Plugs of intact *C. cinereus* chromosomes were prepared as previously described (Muraguchi and Kamada 1998) and subjected to contour-clamped homogeneous electric field (CHEF) gel electrophoresis with a CHEF Mapper (Bio-Rad) using a 0.9% chromosomal grade agarose (Bio-Rad) running at 1.8 V/cm for the first 120 h with a pulse time of 22 min, followed by 48 h with a pulse time ramping from 8 min to 22 min, and exchanging the running buffer of 0.5× TBE [44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM ethylene diamine tetraacetic acid (EDTA)] daily at 14°C. The gel was stained in EtBr (0.5 µg/ml) for 30 min, destained in distilled water for 1 h, and photographed using a Polaroid system. The gel was blotted onto Hybond-N+ membrane by capillary transfer with 0.4 N NaOH. The blots were hybridized using Gene Images Random-Prime Labeling and Detection System (Amersham Biosciences).

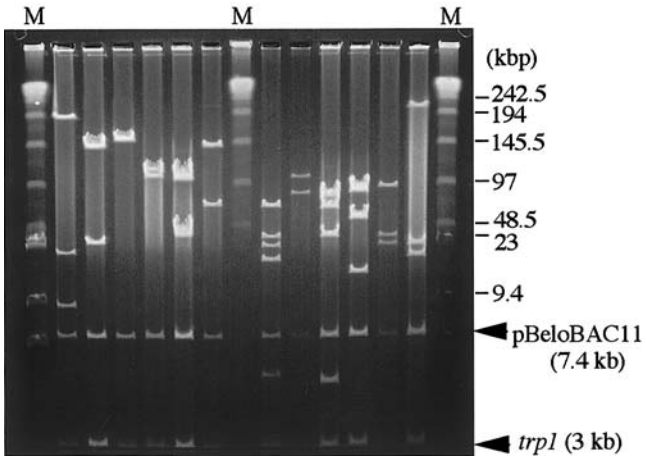

Fig. 2. Contour-clamped homogeneous electric field (CHEF) electrophoresis analysis of BACs. DNAs from 12 clones in the second part of the library (see text) were digested with *Not*I and separated by CHEF electrophoresis (6.0 V/cm; switch time: 5–15 s; run time: 14 h; temperature: 12°C; 1% PFC agarose in 1× TAE). Vector *pBeloBAC11* (7.4 kb) and the *trp1* gene (3.0 kb) are indicated by *arrowheads*. The size markers (*M*) are the λ DNA ladder plus the *Hin*dIII-digested λ DNA; their sizes are indicated in the *right margin*

Results and discussion

A BAC library from *C. cinereus* strain MP#2 was constructed using a novel BAC vector, pBACTZ (Fig. 1). The library consists of 1536 clones (96 well × 16 plates). Strain MP#2 was chosen for the following two reasons. First, most of the chromosomal DNAs of MP#2, especially larger ones, can be separated from each other on a CHEF gel (see Fig. 3, lane 2), allowing us to examine, by Southern hybridization of CHEF blots, which chromosome BAC inserts originate from. Second, because this strain is a member of the mapping progeny used for construction of a linkage map (Muraguchi et al. 2003), it is known from which one of the parents used for the map construction each chromosome in MP#2 comes. These data provide valuable information to search for clones that carry RAPD markers linking to a gene of interest in the BAC library, because some of the RAPD markers are not located on both parents but on one of them.

The genomic DNA was partially digested with *Hin*dIII and then fractionated by CHEF electrophoresis. The size-fractionated DNA was electroeluted from the CHEF gel and drop-dialyzed against 0.5× TE. The drop-dialysis improved ligation efficiency and increased by 20 times the number of recombinant clones (white colonies) obtained in a transformation experiment. One microliter of the 50-µl ligation mixture yielded approximately 100 recombinant clones, representing 5×10^5 colony-forming units (CFU)/µg of vector, independently of the size of fractionated DNA. For a control plasmid (pUC118), efficiencies $>3 \times 10^9$ CFU/µg were routinely obtained. Very few blue colonies were observed in the total transformation experiments, suggesting that the excision of linearized pBACTZ from a gel was effective in eliminating undigested vectors. In addition, no transformant was observed when the electroporation was carried out without polyamines. Polyamines are known to make DNA structures compact (Makita and Yoshikawa 2002) and have been used to construct BAC libraries (Zimmer and Gibbins 1997). Our experiments confirmed that addition of polyamines to DNA solution was essential for *E. coli* transformation with BAC DNAs.

To examine whether the constructed BAC library covers the whole genome of *C. cinereus*, the average insert size of the clones was determined by CHEF electrophoresis of *Not*I digests of BAC clones. The library is composed of two parts: one consists of 1052 clones having ligations of 100–130 kb DNAs and the other of 484 clones having ligations of 160–300 kb. To measure insert size, 53 clones from the former and 75 clones from the latter were analyzed. An example of electrophoresis analysis of BACs from the second part is shown in Fig. 2. It was found that the insert size is 115 ± 21 kb (mean ± SD) in the former and 179 ± 37 kb in the latter. BAC library construction has been reported to suffer from noninsert clones containing small vector fragments (Osoegawa et al. 1998). Such noninsert clones were observed in 7.4% of the clones in the former and 15.4% of those in the latter. Considering the average insert sizes and the percentage of clones with inserts, the entire library constructed in this work is estimated to cover five times the haploid genomes of 37.5 Mb.

To confirm the redundancy of genomic fragments in this library, the whole library was screened by colony hybridization for the presence of ten RFLP markers mapped on nine chromosomes (Table 1). BAC clones were identified for all markers tested. Positive hybridization signals per probe were 5.7 on average, which is good agreement with the estimated redundancy described above.

Two BACs, 13D2 and 13D8, which have inserts of 170 and 182 kb, respectively, were examined for their ability to

Table 1. Evaluation of the *Coprinus cinereus* bacterial artifial chromosome (BAC) library by colony hybridization using ten genomic markers

Marker (gene)	Size[a] (kb)	Chromosome[b]	No. of hits[c]	Hit address[d]									
I-5G4 (*A*-factor)	30	I	5	4A9	5B6	5E12	5G6	11G5					
I398-2F3 (*his5*)	30	I	4	12B8	12F11	14D5	15B2						
Q20-1500B	1.5	II	6	7G3	8A7	11F2	12A8	13A2	14E7				
H3#7	1	III	10	1G6	2E10	4C2	9A11	12A3	12E11	12H3	13C1	13H10	14C2
H5#46	1	IV	3	2H12	8G9	12E1							
A8-800A	0.8	VII	8	3H8	7C6	7D7	9A11	9A12	10A6	12A6	14G1		
H6#25	1	VIII	2	3H4	9B7								
2D5	30	IX	7	3B7	7C3	10A2	12G2	13C2	13E1	14F9			
H7-2#10	1	X	5	2G7	9F5	10D2	11E3	15F7					
219K6 (*benA*)	30	XIII	7	2A3	9G4	10H3	11A9	11H1	13C11	14C12			
Avg.			5.7										

[a] The length of the DNA fragment used as a probe in colony hybridization
[b] The number of the chromosome carrying the marker; the chromosome numbers coincide with those reported (Muraguchi et al. 2003)
[c] Number of clones carrying the marker in the library
[d] The names of clones carrying the marker; the first numeral is the number of a 96-well microtiter plate, and the letter and the last numeral indicate a position in the plate

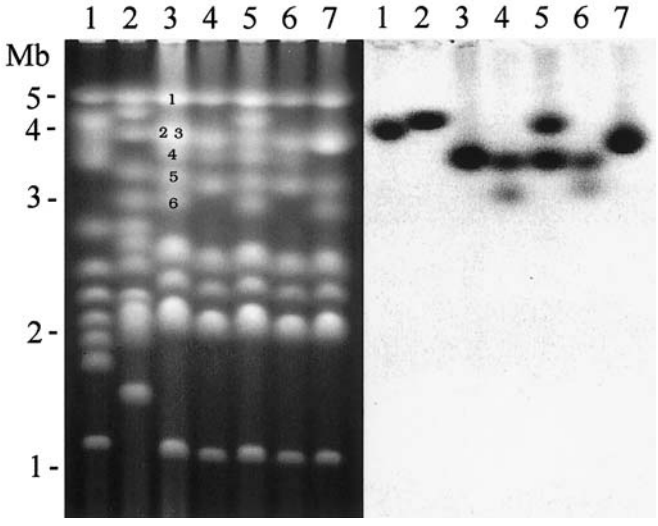

Fig. 3. Electrophoretic karyotypes of the homokaryons, #5302 (*A2B2*), MP#2 (*A91B91*), #292 (*A3B1 trp1-1, 1–6*), and four transformants with the BAC clone, 13D2, carrying a 170-kb insert. *Left panel*: *C. cinereus* chromosomal DNAs separated on a CHEF gel. *Lane 1*, 5302; *lane 2*, MP#2; *lane 3*, #292; *lanes 4–7*, trp$^+$ transformants (#1–#4) with BAC clone 13D2. *Arabic numerals* given on chromosomal DNA bands of #292 represent the chromosome number. *Right panel*: hybridization of the CHEF blot. The CHEF gel in the left panel was blotted, and the blots were hybridized with the probe from the *trp1* gene, which is a selectable marker in BAC vector pBACTZ

transform the tryptophan-requiring *C. cinereus* strain, #292 (*A3B1 trp1-1, 1–6*). Both BACs gave prototrophic transformants at frequencies of ~1.0 CFU/μg DNA. The efficiency of transformation with the BACs was about 100 times as low as those with cosmid DNAs containing 35- and 40-kb inserts in LLC5200 vector (Pukkila and Casselton 1991). However, the BACs usually produced more than 10 trp$^+$ transformants in a single transformation experiment. This transformation efficiency should be sufficient to examine for the ability of a single BAC to complement a mutant phenotype in map-based cloning experiments.

Karyotypes of four transformants by BAC clone 13D2 were examined with CHEF electrophoresis. All transformants examined were different in karyotype from the recipient strain, #292 (Fig. 3, left panel). In transformants #1 and #3, chromosome 6 was lacking (Fig. 3, lanes 4 and 6); in transformant #4, chromosome 4 was absent (Fig. 3, lane 7); in transformant #2, a new chromosome was discernible between chromosome 1 and chromosomes 2, 3 (Fig. 3, lane 5). To examine which chromosome the BAC DNA was integrated into, the CHEF blots were hybridized with a probe from the *trp1* gene, which is a selectable marker in the pBACTZ vector. It was confirmed that the intrinsic *trp1* gene in the recipient strain is located on chromosome 4 (Fig. 3, lane 3). In transformants #1, #2, and #3, the BAC DNA was integrated in chromosome 6, 2 or 3, and 6, respectively (Fig. 3, lanes 4–6). In transformant #4, chromosome 4 at about 3.6 Mb disappeared, a larger chromosome overlapping with chromosomes 2 and 3 was generated, and the larger chromosome gave a strong signal from the *trp1* probe (Fig. 3, lane 7). It would be reasonable to consider that in transformant #4, the BAC DNA was integrated into chromosome 4, causing the enlargement of chromosome 4. Analysis of the four transformants revealed that integration of BACs occurs nonhomologously within the genome and results in the enlargement of the chromosomes with integration. The enlargement of chromosomes suggests that the entire or a large part of the BAC was integrated into the chromosomes. The BAC was preferentially integrated in larger chromosomes. Integration of large DNAs into smaller chromosomes might cause instability of the chromosomes. Low efficiency of the transformation may be the result of the presumed instability of the chromosomes integrated, in addition to insufficient incorporation of BACs with large inserts into protoplasts.

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