A BAC library for *Chlamydomonas reinhardtii*

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We prepared a bacterial artificial chromosome (BAC) library of Chlamydomonas DNA using a modified BAC vector, a plasmid derived from the endogenous *E. coli* F-factor plasmid that accumulates as a circular, supercoiled plasmid with a controlled copy number of 1-2 molecules per cell (Shizuya and Kouros-Mehr, 2001). The BAC vector pBeloBAC11 contains the *lacZ* gene for blue/white color selection of recombinants (Kim *et al*., 1996; Fig. 1). It has a polycloning site with 3 unique restriction enzyme sites within the *lac*Z gene (*Bam*HI, *Sph*I and *Hin*dIII) and these sites are flanked by T7 and Sp6 promoters that can be used to generate RNA probes for chromosome walking. Restriction sites for cutting out the cloned inserts include *Not*I, *Eag*I, *Xma*I, *Sma*I, *Bgl*I, *Sfi*I. The BAC vector contains a chloramphenicol resistance gene for antibiotic selection of transformants.

The bacterial strain used in BAC library construction was DH10B (Durfee et al., 2008). It carries mutations that block the restriction of foreign DNA by endogenous restriction enzymes (*hsd*RMS), and the mutations that block the restriction of methylated DNA (*mcr*A, *mcr*BA, *mcr*C, *mrr*) and recombination (*rec*A). DH10B has high transformation efficiency for large plasmids by using electroporation.

BAC DNA Preparation

The pBeloBAC11 plasmid (7.4 kb) was provided by Drs. Shizuya and Simon of the California Institute of Technology (Kim et al., 1996). A single blue colony from a LB + chloramphenicol (CM) (12.5 μg/ml) + X-GAL (50 μg.ml) + IPTG (25 μg/ml) plate was transferred to liquid medium (3-5 ml of 2XTY + CM) and grown at 37°C overnight with

shaking. An aliquot of this overnight culture was used to inoculate 500 ml of 2XTY + CM (12.5 μg/ml), and the culture was grown overnight at 37°C. The cells were collected by centrifugation at 8000 rpm for 10 minutes in 250 ml bottles in a GSA rotor in a RC2B Sorvall centrifuge. The cell pellet was resuspended in a total of 40 ml of TEG (25 mM Tris pH8, 10 mM EDTA, 50 mM glucose) and kept at room temperature for 5 minutes. A total of 80 ml (2 vol.) of freshly-made 0.2N NaOH, 1% SDS was added and the bottles were inverted several times to mix and then kept on ice for 10 minutes. A total of 60 ml of ice-cold 5M KOAC was added and the bottles were inverted to mix and kept on ice for 10 minutes. The bottles were then centrifuged at 10,000 rpm for 15 minutes at 4°C in the GSA rotor. The supernatant was poured through cheese cloth into a sterile flask and 2 vol. of ethanol were added. After 10 minutes at room temperature, the ethanol precipitate was centrifuged at 8000 rpm for at least 10 minutes. The supernatant was carefully poured off and the precipitate was dried for several minutes in a desiccator. The precipitate was resuspended in 10 ml of TE. Five gm of CsCl and 400 μl ethidium bromide (EB, 10 mg/ml) were added to each 5 ml of resuspended DNA. Cesium chloride density gradient centrifugation was carried out using a TLA100.3 rotor at 75K rpm overnight at 22°C in a TL-100 Beckman ultracentrifuge. The plasmid band was removed from the tubes with a needle and syringe and extracted 5x with equal volumes of NaCl-saturated isopropanol (IPA). Three volumes of water were added to the recovered extracted plasmid and 0.06 new volume of 7.5M NH_4Ac was added. To this was added 0.7 new volume IPA and the solution was mixed gently and kept on ice for one hour. The solution was microfuged for 15 minutes at 4°C and the precipitate was washed in 70% ethanol and dried in a Speed Vac (Savant). The pellet was dissolved in TE, heated at 65°C for 10 min and stored at 4°C. Between 40 – 75 μg of vector DNA was harvested from a 500 ml culture using the rich 2XTY growth media.

Cloning of *NAR2/NIT8* gene into the BAC vector

pBeloBAC11 was modified by introducing the Chlamydomonas *NAR2* gene to allow direct selection of transformants in Chlamydomonas (Galván *et al*., 1996). The *NAR2* gene is the site of the genetic lesion in *nit8* (Nelson and Lefebvre, 1995). The *NAR2* gene construct was isolated as a 4.9 kb *Eco*RI-*Xba*I insert from a plasmid containing the *NAR2 NIT1* gene cluster. *The Eco*RI-*Xba*I insert was blunted with Klenow fragment and *Xho*I linkers were added. After digestion with *Xho*I, the 4.9 kb *Xho*I insert was cloned into the *Xho*I site of pBluescript. The high copy number pBluescript plasmid was used as an intermediate step in the cloning so that more of the 4.9 kb *Xho*I insert could be isolated and because pBluescript allows blue/white color selection for cloning. The 4.9 kb *Xho*I insert cut from pBluescript was ligated into pBeloBAC11 that had been cut with *Xho*I and dephosphorylated with HK phosphatase (Epicentre). After transformation into DH10B cells, colony lifts were performed using the original 4.9 kb *EcoRI-Xba*I insert as the probe to find the new 12.3 kb pBeloBAC11 + *NAR2* plasmid now identified as pBACMN1. Sequencing of the pBACMN1 cosmid revealed the presence of the *NAR2* gene insert that extends from nt 6,993,482 to nt 6,998,480 of the JGI ver5.5 sequence. A link to the DNA sequence is ?????

Preparation of high molecular weight Chlamydomonas DNA

The cell wall-less mutant cw92 (strain CC-503 from the Chlamydomonas Resource Center) was used, the same strain that was sequenced at the JGI. Cells were grown on TAP-0.2M mannitol plates and then collected and washed once in TAP-0.4M mannitol. The cells

were collected by low speed centrifugation in a tabletop clinical centrifuge. The washed cells at a concentration of 1-1.5 x 10^9 cells/ml were gently mixed with liquid 3% LMT agarose (Seaplaque) maintained at 42°C to a final concentration of 0.75% agarose. The agarose was pipeted into 240 μl plug molds which were then kept on ice for 30 minutes to allow the agarose to solidify. Each agarose plug was removed from the mold and cut into two pieces. Each half plug was placed in a well of a 24 well cell culture dish and incubated with 2 ml of ESP (0.25 M EDTA, 1% Sarkosyl, 25 mg/ml proteinase K, 10 mM Tris-Cl pH8) to lyse the cells and digest cell proteins. The agarose plugs were incubated at 50°C without agitation for two days with several changes of ESP. The ESP was removed and 2 ml of 0.05 M EDTA, pH 9 was added and the plugs were incubated at 50°C for one hour. The plugs were then incubated in 2 ml of 0.05 M EDTA, pH 8 for one hour on ice and finally stored in 0.05 M EDTA, pH 8 at 4° C. To prepare for restriction digests of the DNA, the agarose plugs were rinsed in cold TE, incubated in 20 vol of TE + 0.1 mM PMSF (to inactivate Proteinase K) 3X, one hour each, and stored in TE at 4°C.

Partial digestion of high molecular weight DNA in agarose plugs with *Hin*dIII

Each 240 μl plug was divided into 4 pieces for digestion with restriction enzymes. Each piece was cut into 2-4 or more slices with a sterile glass coverslip and the slices were placed in 1.5 ml microfuge tubes with cold (0°C) TE to cover. The TE was removed with a sterile plastic pipet and 0.5 ml of the appropriate buffer for the restriction enzyme was added to each tube. The plugs were incubated 1 hour on ice to allow the buffer to penetrate the agarose plug. The restriction enzyme buffer was replaced with fresh buffer and the agarose plugs were incubated for an additional hour on ice. The buffer was removed and 200 μl of a restriction digest "cocktail" (195 μl of 1x restriction enzyme buffer, 4 mM spermidine) was added to each tube.

After 1 hour on ice, 5 μl of serial dilutions of *Hin*dIII (from 0-10 units) in 1x restriction buffer were added and the solution was gently pipeted up and down to mix without disturbing the DNA-containing agarose. The sample was incubated on ice for 30 min to allow the enzyme to penetrate the agarose and then incubated at 37°C for 30 minutes. To stop the reaction, the tubes were placed on ice and 1/10 vol. (20μl) of 0.5 M EDTA, pH8 was added. After 30 min the EDTA solution was removed and replaced with cold TE. The agarose slices were melted at 65°C for 15 min before being loaded on a CHEF gel. A series of digestions were performed with different amounts of *Hin*dIII to generate partial digestion products of the desired size (100-300 kb). The optimal concentration range of *Hin*dIII was 1-1.5 U of *Hin*dIII per 1/4 plug.

Size selection of DNA fragments using PFGE

Partially digested Chlamydomonas DNA was separated by electrophoresis on a 1% agarose PFG gel in 0.5x TBE. For preparative gels, partially digested Chlamydomonas DNA was run on a 1% LMT agarose (Seaplaque) CHEF gel in 1x TAE. The melted agarose slices of Chlamydomonas DNA cut with 1U of *Hin*dIII per 1/4 agarose plug were loaded on a 100 ml LMT agarose gel in 1x TAE with double-comb wells. Four digests per plug were loaded in each double-comb well. Lambda concatamers (Biorad) and PFGE markers (New England Biolabs) for later staining with EB were loaded on outside lanes of the gel. The gel was run on a CHEF apparatus (DR2 from Biorad) using 2 liters of TAE at 12°C at 6 volts/cm (200V) with a 90 second pulse for 20 hours. Only marker lanes were stained in EB and photographed under UV light. Slices of agarose lanes with digested DNA in the size range of 300-500 kb were cut out with a sterile glass coverslip. Each gel slice was placed in a microfuge tube and rinsed in cold TE twice. The TE was removed and the slice of agarose was stored at 4°C. A small section of

each lane was run on a second PFG under the same conditions to confirm the size of DNA fragments in each gel slice before the DNA was used in a ligation reaction.

Ligation and Transformation

The modified vector pBACMN1 was digested with *Hin*dIII and dephosphorylated with HK phosphatase (Epicentre) as follows: pBACMN1 was cut to completion with *Hin*dIII in NEB2 (New England Biolabs) restriction buffer. The digestion was then treated with RNase, extracted with phenol:chloroform (1:1) and ethanol precipitated and redissolved in TE buffer. The cut vector was incubated in 1x TA buffer (Epicentre) and 5 mM $CaCl_2$ and then treated with HK phosphatase at 1U/ug of plasmid at 30°C for 1 hour. A second 1 U/ug of HK phosphatase was added and the digest was incubated at 30°C for another hour. The HK phosphatase was then heat-killed by incubation at 65°C for 30 minutes.

For ligation reactions, agarose plugs containing HMW DNA were melted at 65°C for 15 minutes and treated with Gelase (Epicentre) at 1U Gelase/100 mg gel slice at 45°C for 1 hour. The concentration of DNA in the liquid agarose was estimated by running an agarose gel with a known concentration of lambda DNA and an aliquot of the Chlamydomonas DNA in liquid agarose. A 50ul ligation reaction was set up using a vector:insert molar ratio of 10:1. Dephosphorylated *Hin*dIII-cut pBACMN1 was incubated with Chlamydomonas DNA at 65°C for 5 minutes and then placed on ice. To avoid shearing HMW DNA, the ends of pipets tips were cut off before use. 10x T4 DNA ligase buffer (final 1x), freshly-made 10mM ATP (final 1mM) and 2μl of ligase (Boehringer Mannheim 2U/μl) were added and the reaction was gently mixed and incubated at 16°C overnight. Then the ligation reaction was incubated at 65°C for 20 minutes to heat-kill the ligase.

Transformation was carried out by electroporating the ligation reaction into DH10B competent cells. The ligation reaction was first drop-dialysed against TE buffer using Millipore VSWP 025 membranes. The dialysed ligation reaction (1-2µl) was used to transform 20ul of *E.coli* ElectroMAX DH10B cells (Gibco-BRL) by electroporation (Transporator Plus, BTX) using 1mm cuvettes at 1.3 kV with a 5 msec pulse. Transformed cells were immediately placed into 0.75 ml of room temperature SOC media and incubated with shaking at 225 rpm at 37°C for 1 hour. The cells were plated on LB + CM + X-GAL + IPTG fresh plates and incubated for at least 24 hours for the blue/white color to develop. White colonies were used to start 3ml LB + CM cultures for plasmid minipreps. The cultures were grown overnight with shaking at 37°C. In the morning, 1.5 ml of culture was used in a modified alkaline lysis plasmid miniprep to give a final volume of 20 ul plasmid prep. (low copy number plasmid). The plasmid minipreps were cut with restriction enzymes (eg. *Not*I, *Bam*HI, *Hin*dIII) to estimate the size of the inserts. The restriction digests were run on a FIGE or agarose gel with lambda *Hin*dIII markers and NEB PFGE markers.

Arraying the BAC Library

To prepare an arrayed library of BAC clones, white colonies were picked robotically (Genome Systems) and arrayed into 40 384-well dishes. Copies of the BAC library were frozen in media containing 15% glycerol, covered with Biomek Seal & Sample Aluminum Foil Lids (Beckman Coulter) and maintained at -80^{o}C.

Alignment of BAC clones with markers on Chlamydomonas chromosomes

Molecular markers aligned on the Chlamydomonas genetic map (Kathir et al., 2013) were used to generate BAC clone contigs aligned with the map (https://www.chlamycollection.org/resources/maps/bac-maps/). The BAC clones were transferred to nylon filters. DNA probes corresponding to molecular markers were random primer labeled with ^{32}P and hybridized to BAC clone DNA on the filters. BAC clones that hybridized to the probes were picked from frozen stocks for DNA isolation in a 96-well format (Sarstedt).

BAC Minipreps and contig construction

This method is based on an alternative method described in Marra *et al*., 1997.

Day 1-2: Each frozen stock was streaked onto LB + CM agar medium and a single colony was picked into a well containing 150 µl LB + CM. The precultures were grown 16-18 hr at 37°C without shaking. After covering the plate with AirPore tape (Qiagen) it was vortexed gently using a Thermolyne flat vortexer.

Day 3: A Boekel Replicator (Fisher Scientific) was used to inoculate the cultures into 1.3 ml of 2XYT + CM growth medium contained in 2-ml deep wells of replicate 96-well blocks (Polyfiltronics). The replicate blocks were carried through the culture growth and DNA isolation steps below and the DNA from replicate wells was combined in the filtration step. The precultures were frozen by addition of 75 µl of each preculture to a well in a second 96-well dish containing 75 ul of 30% glycerol. The plates containing the glycerol stocks were sealed with aluminum foil tape and placed in a -80°C freezer. The inoculated blocks were sealed with AirPore tape and the cultures were grown at 37°C with shaking at 300 rpm for 18 hr.

Day 4: After cooling on ice for 15 min, the blocks were centrifuged at 3500 rpm for 20 min at 4°C in a Hermle Z 383 K centrifuge. The media was poured off and the blocks were blotted briefly on paper towels. An alkaline lysis procedure was used for DNA isolation. To each well was added 150 μl Soln I (25 mM Tris, pH8.0, 10 mM EDTA, 50 mM glucose, 0.12 mg RNAse/ml added just before use). The blocks were sealed with aluminum tape (Scotch Brand), vortexed to fully resuspend each cell pellet, and incubated at RT for 30 min. The seal was removed and 150 μl of Soln II (0.2N NaOH, 1% SDS), prepared just before use, was added to each well. The blocks were sealed, inverted gently 20 times, and incubated at RT for 5 min. The seal was removed and 150 ul ice-cold Soln III (5 M KOAc) was added to each well. The blocks were sealed, inverted 10 times to mix, incubated on ice for 10 min, and centrifuged at 4000 rpm at 4°C for 15 min. The supernatants from two replicate blocks (800 ul total) were filtered through 96-well UNIFILTER filtration plates with 0.45 μm cellulose acetate filters into a 96-well 2 ml block using a vacuum manifold at -7 psi (Whatman Polyfiltronics Vacuum Assist Frame). DNA was precipitated by adding 330 μl 100% ethanol to each well, sealing the block, and inverting the block 3 times. After incubation at RT for one hr, the block was centrifuged at 4000 rpm for one hr at RT in the Hermle centrifuge. The ethanol was decanted by inverting the plate and the pellets were washed twice by adding 500 μl of 70% ethanol and inverting the plates on paper towels. The pellets were dried under vacuum for one hr. The pellets were resuspended by adding 30 μl 10 mM Tris, pH 8, to each well, covering the block with foil tape, and incubating overnight at 4°C.

Day 5: The block was centrifuged at 1000 rpm for 5 min at 4°C, vortexed gently, and centrifuged again. Optical density reading of the DNA indicated a yield of 1 to 2 μg DNA. For sequencing the BAC DNA ends, a 10 ml aliquot was used along with 10 pmol T7 or SP6 primer.

For groups of BAC clones that hybridized to a common molecular marker probe, contigs were constructed by comparing the restriction fragments shared among the BAC DNAs run on the same gel. Aliquots of 20 µl of the BAC DNA prep were digested with *Bam*HI and *Hin*dIII (10 units each) in 30 µl reactions for 5 hr at 37°C. From each reaction, 20 µl was electrophoresed on a 13 x 20 cm gel prepared from 150 ml 1.5% agarose in TAE buffer. The gel was run in at cold room (8°C) in a Jordan Scientific gel apparatus at 55 V for 18 hr with buffer recirculation. The gel was stained with ethidium bromide (10 µg/ml) for 30 min and destained in H_2O at 4°C for 18-24 hr. A photograph of the destained gel was used to compare the restriction fragments in different BAC DNA samples. Overlapping clones were arranged into contigs with an average size of 100kb. https://www.chlamycollection.org/resources/maps/bac-maps/

To isolate BAC DNA for transformation, we recommend using a BAC DNA isolation kit, such the FosmidMAX kit (Lucigen Biotechnologies).

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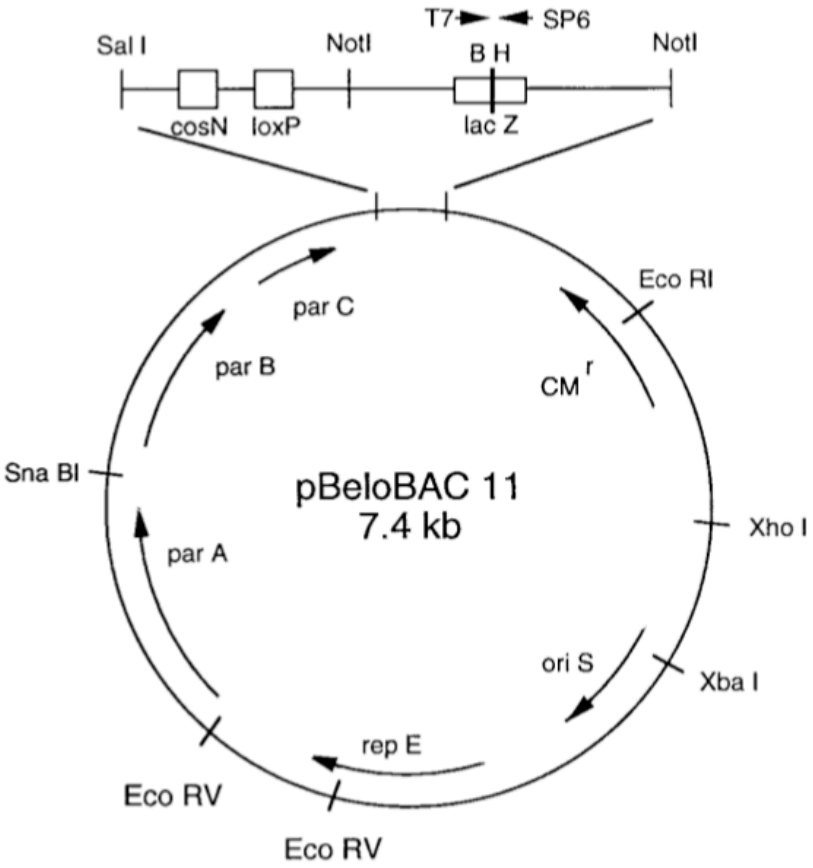

FIG. 1. Diagram of pBeloBAC11 vector. The lacZ gene fragment was introduced to the *Not*I site of pBAC108L. Insertional inactivation of the gene by a cloned DNA fragment results in white colonies on X-gal/IPTG plates after transformation.

This figure is from Kim et al., 1996. A 4.9 kb fragment containing the *NAR2* gene was cloned into the *Xho*I site of pBeloBAC11 to generate the 12.3 kb pBACMN1 plasmid.