

Construction of Small Genome BAC Library for Functional and Genomic Applications

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1. Introduction

The use of genetic approaches to study bacteria is limited by the following considerations: first, the bacteria must be easy to culture and nonpathogenic; and, second, a broad spectrum of genetic techniques for the particular bacteria being studied must be available. For other bacteria, such as the syphilis-causing spirochete, *Treponema pallidum*, genetic studies are highly limited because the bacterium cannot be continuously grown in the laboratory, because of its high virulence and a total lack of genetic tools to study this organism.

Construction of genomic libraries represents a powerful resource for genetic studies of bacteria including nonculturable organisms. For example, screening of a library for specific functions can be used for identification of genes coding for antigens, exported proteins, enzymes, receptors, regulators, and other activities.

The bacterial artificial chromosome (BAC) cloning system was invented for cloning of large fragments (80–300 kb) of eukaryotic DNA. However, there are several examples of using this technology for bacteria (1–4). In contrast to eukaryotic BAC libraries, gene expression from inserts of bacterial DNA in BACs was detectable and the functions of expressed genes can be studied (4,5). This expression is likely from bacterial transcription and translation signals in the insert and does not require special vector sequences. The major advantage of the BAC vector, derived from the F plasmid, is the strictly controlled copy number in *Escherichia coli*. The BAC plasmid is present in one to two copies per cell, which is important for genes that are toxic when overexpressed. Owing to the low BAC copy number, the insert length that can be recovered in BAC clones is usually much larger than for other cloning systems. BAC clones thus

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can be used for construction of libraries covering the bacterial genome with a relatively small number of *E. coli* clones. Minimal sets of clones covering the bacterial chromosome can be subsequently used for strain comparisons, experiments in functional genomics, and genomic applications.

2. Materials

2.1. Preparation of Chromosomal DNA

1. Tris/EDTA (TE) buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
2. Low-melting-point InCert agarose (FMC BioProducts, Rockland, ME).
3. TE buffer supplemented with 0.5% sodium dodecyl sulfate (SDS).
4. Proteinase K (Sigma, St. Louis, MO).

2.2. Partial Digestion of *T. pallidum* Chromosomal DNA

1. Triton X-100 (0.1%).
2. *Hind*III restriction endonuclease and *Hind*III buffer 2 (New England Biolabs, Beverly, MA).
3. 50 mM EDTA (pH 8.0).

2.3. Pulsed-Field Gel Electrophoresis of Digested DNA, Size Selection, and Electroelution

1. I.D.NA[®] agarose (BioWhittaker, Rockland, ME).
2. DR II apparatus (Bio-Rad, Hercules, CA), for electrophoresis by the contour-clamped homogeneous electric field (CHEF).
3. 0.5X Tris/acetate (TAE) buffer: 20 mM Tris-acetate, 0.5 mM EDTA, pH 8.3.
4. 1X TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.3.
5. λ DNA ladder (BMA, Rockland, ME).
6. 0.5 M EDTA (pH 8.0).
7. Dialysis tubing ($\frac{1}{4}$ - to $\frac{3}{4}$ -in. diameter) (Life Technologies, Gaithersburg, MD).

2.4. Preparation and Digestion of Vector DNA

1. *E. coli* VCS257 (Stratagene, La Jolla, CA) carrying pBeloBAC11 (6).
2. Luria Bertani (LB) plates containing 25 μ g/mL of chloramphenicol, 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and 40 μ g/mL of 5-bromo-4-chloro-3-indolyl- β -galactoside (X-Gal).
3. LB medium containing 25 μ g of chloramphenicol/mL.
4. Qiagen Plasmid Kit for isolation of BAC DNA (Qiagen, Valencia, CA) containing buffers P1, P2, P3, QBT, QC, QF, and Qiagen-tip 500 column.
5. P1 buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μ g/mL of ribonuclease A (Sigma).
6. P2 buffer: 200 mM NaOH, 1% SDS.
7. P3 buffer: 3.0 M potassium acetate, pH 5.5.
8. Isopropanol and 70% ethanol.

9. *Hind*III endonuclease and calf intestinal alkaline phosphatase (CIP) (New England Biolabs).
10. Ultrapure agarose (Gibco-BRL, Life Technologies, Gaithersburg, MD).
11. TBE buffer: 89 mM Tris-borate, 2 mM EDTA, pH 8.3.
12. QIAquick Gel Extraction Kit (Qiagen).

2.5. Ligation, Dialysis, and Electroporation

1. T4 DNA ligase (New England Biolabs).
2. VSWP 0.025- μ m membranes (Millipore, Bedford, MA).
3. Electrocompetent ElectroMAX DH10B cells (Life Technologies).
4. LB medium.
5. Filter-sterilized glycerol (10%).
6. Gene Pulse Controller II apparatus (Bio-Rad) and 0.2-cm gap electrode cuvetts.
7. SOC medium: 2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose.
8. Chloramphenicol-containing LB plates (12.5 μ g/mL) supplemented with 0.5 mM IPTG and X-Gal (40 μ g/mL).

2.6. Isolation of DNA From Individual BAC Clones

1. Qiagen Plasmid Kit for isolation of BAC DNA (Qiagen), Qiagen-tip 20 columns.
2. LB medium supplemented with 12.5 μ g/mL of chloramphenicol.

2.7. End Sequencing and Restriction Mapping of BAC Clones

1. *Hind*III, *Eco*RI, *Xba*I, and *Xho*I restriction endonucleases with corresponding reaction buffers (New England Biolabs).
2. Model 377 DNA sequencing system (Applied Biosystems, Foster City, CA).
3. ABI Prism® BigDye™ Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems).
4. Polymerase chain reaction (PCR) primers with target sites on pBeloBAC11 (GW386: 5'-TTGTAAAACGACGGCCAGTG-3'; GW387: 5'-TTACGCCAAGC TATTTAGGTGAC-3').

3. Methods

3.1. Preparation of Chromosomal DNA

1. Grow *T. pallidum* subspecies *pallidum* (Nichols) strain in rabbit testes, harvest, and purify the cells using sodium diatrizoate gradient centrifugation (7,8). Wash the *T. pallidum* cells four times, and resuspend in TE buffer to a concentration of 2×10^{10} cells/mL.
2. Mix an equal volume of *T. pallidum* cells with molten 1.6% low-melting-point InCert agarose, and apply 200 μ L of this mix into plug molds and allow solidification.
3. Gently remove the resulting $15 \times 9 \times 1.5$ mm plugs, and put into 30 mL of TE buffer supplemented with 0.5% SDS and incubate overnight at 37°C. Subse-

quently, add proteinase K to a final concentration of 100 µg/mL and incubate the plugs for an additional 48 h at 55°C. Then wash the plugs four times with TE buffer for 60 min each.

3.2. Partial Digestion of *T. pallidum* Chromosomal DNA

1. Partial digestion is performed according to Brosch et al. (1). Wash the chromosomal DNA-containing plugs three times with 0.1% Triton X-100, and then equilibrate three times in 10 mL of *Hind*III buffer 2 supplemented with 0.1% Triton X-100 for 60 min at 4°C.
2. The last equilibration step is done on ice. After removal of the buffer, transfer each plug to 1 mL of ice-cold buffer 2 containing *Hind*III restriction endonuclease (20 U/mL) and incubate for 2 h on ice. Then incubate the plugs for 30 min at 37°C (see Note 1).
3. Stop digestion by adding 0.5 mL of 50 mM EDTA (pH 8.0) to 1 mL of *Hind*III-containing buffer 2.
4. Use the plugs with digested DNA for fragment separation by pulsed-field gel electrophoresis (PFGE).

3.3. PFGE of Digested DNA, Size Selection, and Electroelution

1. Place the plugs with partially digested DNA into the wells of a 1% I.D.NA agarose gel, and subject to electrophoresis by the CHEF method using the DR II apparatus. Prepare and run the gels in 0.5X TAE buffer. Perform PFGE at 14°C and 6 V/cm for 16 h with a 5- to 45-s pulse time at a 120° angle. A λ DNA ladder is used for size markers from 48.5 kb to more than 1 Mb.
2. Remove the marker lanes from the gel and stain with ethidium bromide to visualize the positions of individual marker bands. Then cut out the lanes containing digested genomic DNA in the region corresponding to DNA fragment lengths of 40–200 kb, and divide into an additional four agarose blocks containing fragments with 40–80, 80–120, 120–160, and 160–200 kb, respectively. The size selection of digested *T. pallidum* DNA is shown in Fig. 1. For cloning of DNA fragments, digested DNA is exposed neither to ethidium bromide nor to ultraviolet light (see Note 2).
3. Directly use gel slices for electroelution or store in 0.5 M EDTA (pH 8.0) at 4°C.
4. Electroelution of digested genomic DNA from gel slices is performed according to Strong et al. (9). Equilibrate the gel slices with 1.0X TAE buffer for 3 h and subsequently transfer into dialysis tubing of 1/4- to 3/4-in. diameter (Life Technologies) with 200–400 µL of fresh 1.0X TAE buffer. Adjust the gel slice to one side of the dialysis tubing with the slice long axis perpendicular to the voltage gradient and put closer to the negative electrode. Elute the DNA from the gel at 2.5 V/cm for 2 h, and at the end of elution, reverse the polarity of current for 30 s to detach the DNA molecules from the bag.
5. Carefully remove the eluted DNA with a wide-bore pipet tip and either use directly for ligation or store at 4°C (see Note 3).

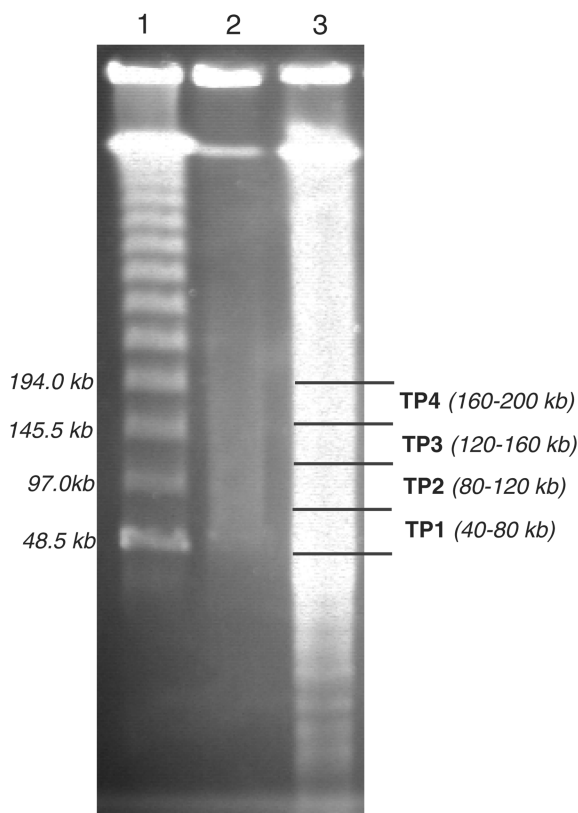


Fig. 1. PFGE of undigested and *Hind*III-treated *T. pallidum* chromosomal DNA. Lane 1, λ DNA ladder (48.5 kb to more than 1 Mb); lane 2, *T. pallidum* chromosomal DNA undigested; lane 3, *T. pallidum* chromosomal DNA digested for 30 min with *Hind*III restriction endonuclease. Note the four regions, TP1–TP4, of the gel used for excision of size-separated DNA fragments.

3.4. Preparation and Digestion of Vector DNA

1. The pBeloBAC11 vector (6) is used for construction of the library. Inoculate *E. coli* VCS257 carrying pBeloBAC11 on an LB plate containing 25 μ g/mL of chloramphenicol, 0.5 mM IPTG, and 40 μ g/mL of X-Gal and incubate overnight at 37°C. Use a single blue colony for inoculation of 1 L of LB culture containing 25 μ g of chloramphenicol/mL of medium.
2. Harvest the cells and isolate the plasmid DNA using a modified Qiagen Plasmid Protocol for isolation of BAC DNA (Qiagen). Carefully resuspend the cells to 100 mL of P1 buffer at 4°C until no cell clumps are visible. Add 100 mL of room

temperature P2 buffer, gently mix with the cells resuspended in P1, and incubate at room temperature for 5–10 min to lyse the cells. After visible lysis, add 100 mL of prechilled buffer P3, incubate the mixture on ice for 10 min, and centrifuge twice at 20,000g for 20 min at 4°C, discarding the sediment. Apply the clear supernatant to a QBT buffer–equilibrated Qiagen-tip 500 column, wash twice with QC buffer, and elute the vector DNA from the column with QF buffer prewarmed to 60°C according to the manufacturer’s recommendations.

3. Precipitate the eluted DNA with 0.7 vol of room temperature isopropanol, and immediately centrifuge at 15,000g for 30 min at 4°C. Discard the supernatants, wash the DNA with 70% ethanol, and recentrifuge the DNA for 15 min at 15,000g at 4°C. Remove the ethanol and dry the DNA pellet for 5 min in a vacuum.
4. Resuspend the DNA in 100 μ L of distilled water and measure the DNA concentration. Digest the pBeloBAC11 plasmid (100 ng) with 5 U of *Hind*III for 2 h at 37°C, dephosphorylate by adding 5 U of CIP, and incubate for 30 min at 37°C. Run the digested vector DNA on a 1% agarose gel in TBE buffer, and cut out the DNA band corresponding to the digested vector DNA, and extract from the gel using the QIAquick Gel Extraction Kit, and measure the DNA concentration (see **Note 4**).

3.5. Ligation, Dialysis, and Electroporation

1. Ligate 10 ng of size-selected *T. pallidum* DNA to 1 ng of *Hind*III-digested and -dephosphorylated pBeloBAC11 DNA overnight at 16°C with 200 U of T4 DNA ligase.
2. Inactivate T4 DNA ligase at 65°C for 10 min, and then drop-dialyze the ligation solution against TE buffer using VSWP 0.025- μ m membranes.
3. Prepare electrocompetent ElectroMAX DH10B cells after harvesting of an LB culture at $OD_{600} = 0.5$. Then extensively wash the cells five times with ice-cold water and resuspend in 10% ice-cold filter-sterilized glycerol to an OD_{600} of approx 100, and either directly use for electroporation or store as 50- μ L aliquots at –80°C.
4. Mix 50 μ L of competent cells with 1 μ L of ligation mixture in a 0.2-cm gap electrode cuvet on ice. Use a Gene Pulse Controller II apparatus set to 2.5 kV, 25 μ F, and 100 Ω .
5. Immediately after electroporation, add 0.6 mL of sterile SOC medium prewarmed to 37°C and grow the cells for 1 h at 37°C with shaking at 100 rpm.
6. Plate the cells on chloramphenicol-containing LB plates (12.5 μ g/mL) supplemented with 0.5 mM IPTG and X-Gal (40 μ g/mL). Incubate the plates for at least 24 h at 37°C or, for better results, for more than 48 h. Isolate the white colonies and use for further investigations (see **Note 5**).

3.6. Isolation of DNA From Individual BAC Clones

1. Use white colonies for isolation of clone DNA (see **Note 6**).
2. Use the same, but scaled down, procedure as for isolation of pBeloBAC11 DNA. Inoculate each white colony into 10 mL of LB medium with 12.5 μ g/mL of chlo-

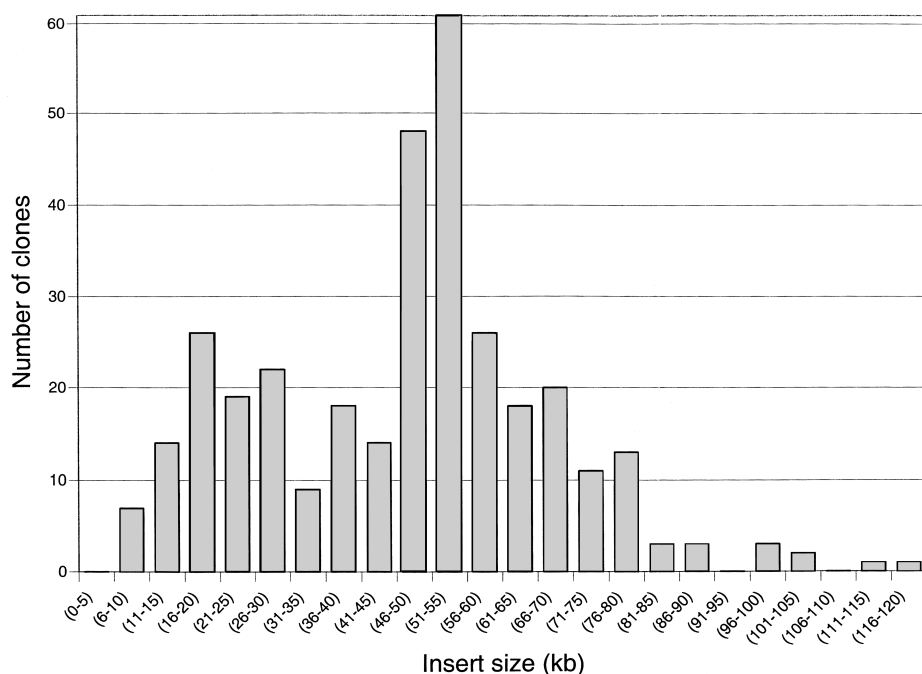


Fig. 2. Distribution of insert lengths of 339 clones in the library. Numbers of clones containing insert sizes in 5-kb increments are shown starting with 0–5 kb. The majority of the clones contained insert sizes of 45–50 and 50–55 kb. The largest insert obtained slightly exceeded 120 kb. A second peak for the insert lengths of 10–30 kb indicates preferential cloning of small inserts, probably as a result of higher cloning, ligation, and/or transformation efficiency.

ramphenicol, and after overnight incubation, extract the DNA using 2 mL of P1, P2, and P3 buffers and Qiagen-tip 20.

3. Resuspend the dried DNA pellet in 20 μ L of distilled water and store at -20°C .

3.7. End Sequencing and Restriction Mapping of BAC Clones

1. Use the isolated clone DNA (4 μ L) for an initial *Hind*III restriction digestion analysis to test for the presence of the insert (see **Note 7**).
2. Use the clone DNA (9 μ L) as a template for DNA sequencing reactions. Sequence the DNA using the *Taq* Dye-deoxy Terminator method and a model 377 DNA sequencing system. Use two PCR primers with target sites on pBeloBAC11 to sequence both insert termini, GW386 and GW387.
3. Align the DNA sequence obtained with the *T. pallidum* whole genome sequence (10) and map the position and length of each clone. No noncontiguous clone sequences were found. The distribution of insert lengths is shown in **Fig. 2**.

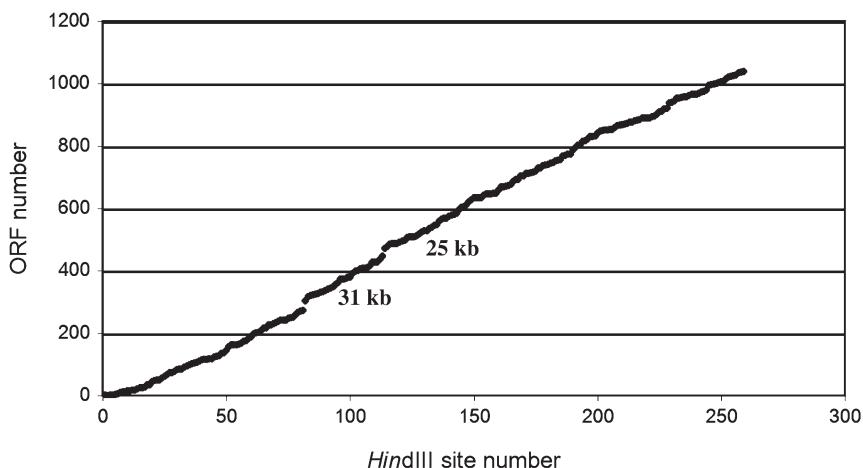


Fig. 3. Distribution of *Hind*III target sites along *T. pallidum* chromosome. For each individual *Hind*III site, the corresponding ORF containing the target site is plotted. The 259 *Hind*III sites are randomly distributed through 1040 predicted *T. pallidum* ORFs. The two largest regions without *Hind*III sites (31 and 25 kb) are indicated.

4. Additionally map selected clones with *Hind*III, *Eco*RI, *Xba*I, and *Xho*I restriction endonucleases, and compare the fragments obtained with those predicted (see Note 8).

4. Notes

1. The *Hind*III restriction endonuclease was chosen for construction of the *T. pallidum* library because of the unique cloning site on the pBeloBAC11 vector (6) and random distribution of 259 individual *Hind*III target sites throughout the *T. pallidum* chromosome (Fig. 3). Large regions without *Hind*III target sites would prevent their cloning and presence in the library. The two largest regions of the *T. pallidum* chromosome without *Hind*III target sites are a 31-kb region between bp 288,534 and 316,779 (open reading frame [ORF] TP0273–TP0304) and a 25-kb region between bp 474,463 and 499,924 (ORF TP00448–TP0471; Fig. 3). Both of these are small enough to be clonable in a large-insert (>30 kb) library.
2. Preelectrophoresis and elimination of small fragments can be used to achieve more homogeneous distribution of large inserts (11).
3. Alternatively, isolation of DNA fragments from agarose blocks can be performed by enzyme digestion of agarose with Gelase (Epicentre, Madison, WI) after agarose is melted for 10 min at 65°C (1). However, electroelution was shown to yield more intact DNA than isolation by the agarose digestion treatment (9). To our knowledge, electroelution is gentler and more efficient and thus more suitable for isolation of DNA fragments in which the source of DNA is limited.

4. A high-quality preparation of vector DNA can be used to achieve a reduced background of nonrecombinant clones (11).
5. Increased electroporation efficiency can be achieved by the method published previously (12).
6. Most of the white colonies were obtained for the DNA fraction with fragment lengths between 40 and 80 kb. Approximately 10% of all white colonies isolated were obtained with ligated DNA fragments 80–120 kb, and two fractions with an insert size of 120–200 kb repeatedly resulted in only clones with no inserts. A similar observation was made for construction of a *Mycobacterium tuberculosis* large-insert library in which human DNA was used as a positive control. The maximum size of the insert for prokaryotic DNA was shown to be considerably lower than for eukaryotic DNA (1).
7. Approximately 20% of the white colonies contained no insert clones and were discarded.
8. In 2 of 26 (7.7%) clones investigated, deletions of more than 30 kb inside the insert were observed. However, the clones used for restriction mapping were not selected randomly; that is, only the largest clones along the *T. pallidum* chromosome were used. These data, together with the finding that the cloned *T. pallidum* DNA fragments in the library were unequally distributed throughout the *T. pallidum* chromosome (not shown), suggest that the maximum size and stability of large inserts in BAC vectors depend not only on the prokaryotic vs eukaryotic source, but also on the specific gene content of each clone.

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