

Recovery, Purification, and Cloning of High-Molecular-Weight DNA from Soil Microorganisms[∇]

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We describe here an improved method for isolating, purifying, and cloning DNA from diverse soil microbiota. Soil microorganisms were extracted from soils and embedded and lysed within an agarose plug. Nucleases that copurified with the metagenomic DNA were removed by incubating plugs with a high-salt and -formamide solution. This method was used to construct large-insert soil metagenomic libraries.

Isolation of genomic DNA from soil microorganisms without cultivation (i.e., “metagenomic DNA”) using harsh extraction methods results in DNA that is typically less than 100 kb in size (8, 14, 17, 20, 21, 24). For construction of a metagenomic library from a microbial assemblage, there are advantages in obtaining large contiguous genomic DNA fragments, for example, those that contain intact biosynthetic pathways or can be used to link phylogenetically informative sequences with functionally informative contiguous sequences (4, 17). Here we use an indirect DNA extraction method in which microbial cells were separated from soils (1, 5, 7, 10, 13, 22) to recover high-molecular-weight (HMW) environmental DNA for library construction (2, 9). While this method reproducibly results in isolation of HMW genomic DNA greater than 1 Mbp in size, we observed that with many soils the DNA could not be readily cloned due to the presence of contaminating nuclease activity. Therefore, we sought improvements that would achieve the removal of associated contaminants from HMW environmental DNA embedded within an agarose gel matrix and that would preserve genomic DNA integrity.

We isolated microbial cells from soils at the West Madison Agricultural Research Station (WMARS), the National Science Foundation Long-Term Ecological Research Site at Bonanza Creek Experimental Forest near Fairbanks, AK (BCEF), the Hancock Agricultural Research Station (HARS), the Curtis Prairie at the University of Wisconsin—Madison Arboretum (UW Arboretum), and the Auburn University Arboretum (AU Arboretum). These soils have diverse physical structures, with representatives of high-clay-content (WMARS and AU Arboretum), high-sand-content (HARS), and high-silt-content (BCEF and UW Arboretum) soils (3, 18, 19, 25). The bacterial cells were recovered from each soil using Waring blender homogenization, differential centrifugation, and cell purification

(2, 5, 9, 22). In some soils (WMARS, BCEF, and UW Arboretum), we could enhance the dissociation of bacterial cells from soil particles using sodium deoxycholate, polyethylene glycol, and/or an anion exchange resin (data not shown) (6, 11, 12, 23).

HMW genomic DNA was isolated using a combination of chemical and enzymatic lysis within an agarose plug (9) (Fig. 1). Briefly, extracted and washed bacterial cells were pelleted by centrifugation and embedded within low-melting-point agarose (Promega, Madison, WI) in a 1-ml syringe. The agarose plug was then extruded from the syringe and incubated in 10 ml of lysis buffer (1% Sarkosyl, 1% sodium deoxycholate, 1 mg/ml lysozyme, 10 mM Tris-HCl [pH 8.0], 0.2 M EDTA [pH 8.0], and 50 mM NaCl) for 1 h at 37°C. The plug was transferred into 40 ml of ESP buffer (1% Sarkosyl, 1 mg/ml proteinase K, and 0.5 M EDTA [pH 8.0]) and incubated for 16 h at 55°C, followed by inactivation of proteinase K with 1 mM phenylmethylsulfonyl fluoride from a fresh phenylmethylsulfonyl fluoride stock in isopropanol with 1 h of incubation at room temperature. After three 10-min washes in 10 mM Tris-HCl with 1 mM EDTA (pH 8.0) buffer (T₁₀E₁), plugs were stored at 4°C in 10 mM Tris-HCl with 50 mM EDTA (pH 8.0). By comparison to DNA isolated by direct extraction, the DNA isolated from microbial cells was significantly larger, ranging in size from less than 20 kb to more than 1 Mb, albeit with a lower yield, ranging from approximately 10 to 25% of that achieved by direct lysis (data not shown).

The HMW DNA from each soil was electrophoresed from an agarose plug into CleanCut agarose (Bio-Rad, Hercules, CA). Soil metagenomic DNA could be restriction digested with Sau3AI for all soils, whereas HindIII failed to restriction digest the same DNA. However, nuclease activity was observed in the control reactions that contained soil DNA and restriction buffer (containing 6 mM MgCl₂) at 37°C (Fig. 2, lanes 5 and 6), with a nearly complete loss of DNA observed with some soils (AU Arboretum, BCEF, HARS, and UW Arboretum), preventing DNA cloning.

Numerous methods were employed to remove nuclease contamination. For large-insert cloning, it is imperative to prevent genomic DNA shearing, and it has been reported that electrophoresis of genomic DNA through an agarose gel that includes

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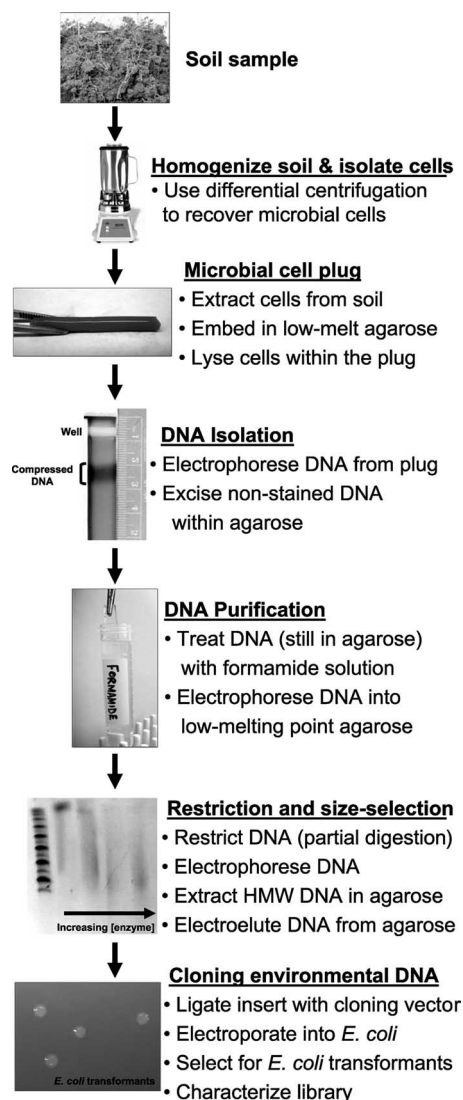


FIG. 1. Flow chart for the recovery, purification, and cloning of HMW metagenomic DNA from soil microorganisms.

polyvinylpyrrolidone could reduce the presence of interfering contaminants (16), but in none of the samples tested was this method effective at removing nuclease activity (data not shown).

The one method that showed a consistent and significant benefit in removing associated nucleases from metagenomic DNA was treatment with a high concentration of formamide and sodium chloride within an agarose plug (Fig. 2, lanes 3 and 4). The formamide serves to denature genomic DNA and associated proteins and other factors bound to DNA, and sodium chloride enhances genomic DNA stability during denaturation (18). The genomic DNA becomes denatured while still embedded within the agarose matrix and then may be renatured by dialysis. To accomplish this denaturation step, agarose plugs containing embedded and lysed cells were placed along the entire top of a 1% agarose gel and DNA was electrophoresed for 4 to 5 h in a cold (4°C) room. After a narrow strip of the gel was stained to determine where the DNA was present (Fig. 1), the unstained portion of agarose (with agar volume kept to a

minimum) containing compressed HMW DNA was then excised and placed within a 15-ml centrifuge tube containing 80% formamide and 0.8 M NaCl in a 20 mM Tris-HCl buffer (pH 8.0), providing a 60 to 70% final formamide concentration. The plug was incubated overnight at 15°C, and then, to remove the formamide and renature DNA, the agarose plug was placed directly into a 1-liter volume of T₁₀E₁ at 4°C and the solution was very gently stirred on a magnetic plate for at least 24 h. After dialysis, the purified agarose plug was placed in a new agarose gel and metagenomic DNA was electrophoresed into low-melting-point agarose for approximately 3 h at 70 V in a cold (4°C) room. The resultant agarose plug of purified HMW DNA was immediately sliced into 4-mm sections, and each section was placed in a microcentrifuge tube containing 500 µl of T₁₀E₁ and stored at 4°C until restriction digested (within 24 h).

To subject DNA within a cell plug section to restriction digestion, between 1 and 10 units of Sau3AI were used per 4-mm cell plug section in a 200-µl volume in 1× restriction buffer and 1× bovine serum albumin for 1.5 h at 37°C. The partially digested HMW DNA was size selected using pulsed-field gel electrophoresis (PFGE) (0.3- to 3-s switch time, 120°, -1.5 ramping factor), and the recovered agarose slice was treated with GELase (Epicenter, Madison, WI), or it was electroeluted from the gel slice (15). In some experiments, multiple size selection gels were employed to reduce the presence of small-molecular-weight DNA in subsequent ligations. The partially digested, size-selected genomic DNA was then ligated into a commercially prepared fosmid or bacterial artificial chromosome (BAC) vector at an approximate molar ratio of 10 insert:1 vector, using T4 DNA ligase overnight at 15°C. The ligation mixture was heat inactivated, dialyzed against double-distilled water, and then electroporated into highly electrocompetent *Escherichia coli* cells. Transformants were selected on LB media containing Cm_{12.5}, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-β-D-thiogalactopyranoside, and white colonies were selected after 15 h of incubation.

Many parameters are important to evaluate for successful large-insert cloning. Besides purity of the HMW DNA, using a cloning vector that has been dephosphorylated to prevent self-ligation, achieving efficient recovery of the HMW DNA by electroelution, and using multiple vector:insert ratios in ligations are critical in any library construction. Each environmental sample has unique challenges, and this protocol helps to remove a critical hurdle (i.e., nuclease contamination) that may prevent large-insert cloning from many soils. While large-insert library construction is to some degree an idiosyncratic process and the specific effect of the formamide treatment has not been identified, there was clearly an increase in the efficiency of library construction when formamide (and high salt) was used to treat the recovered HMW DNA prior to restriction digestion (Table 1). In 21 library construction attempts with the BCEF soil without formamide treatment, a total of approximately 19,000 recombinant clones were obtained, and in 15 of these attempts, no genomic inserts were obtained. In contrast, in 7 library construction attempts using the formamide treatment, more than 475,000 clones were obtained, and in only 1 case were no inserts obtained (Table 1). While some of these library construction attempts were made with fosmid as well as BAC vectors, in the two cloning attempts that utilized

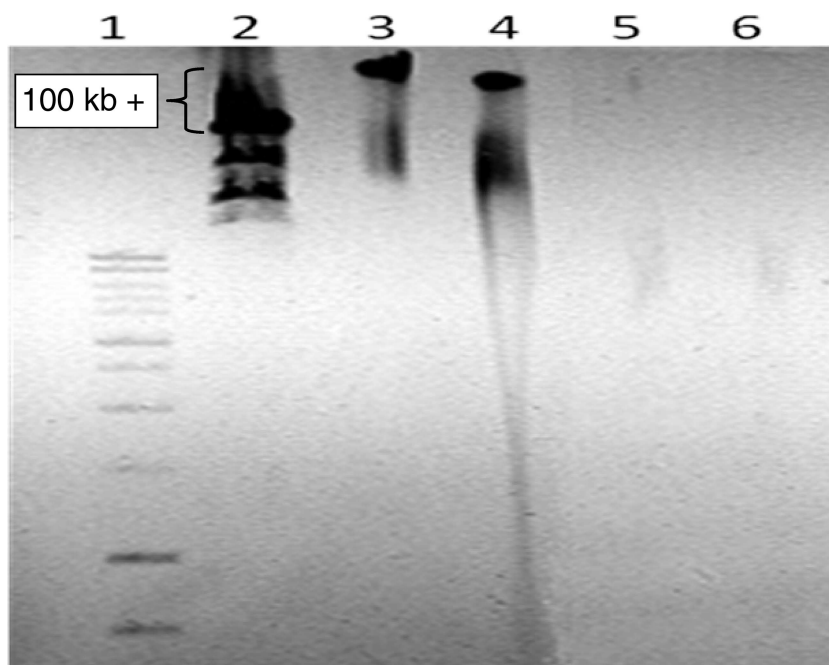


FIG. 2. Formamide treatment of soil metagenomic DNA prevents DNA degradation. Lane 1, 1-kb DNA ladder (Promega); lane 2, MidRange II PFGE marker (New England BioLabs); lane 3, BCFE soil DNA plus formamide and salt treatment; lane 4, BCFE soil DNA plus formamide and salt treatment plus Sau3AI (10 U); lane 5, BCFE soil DNA; lane 6, BCFE soil DNA plus Sau3AI (10 U). PFGE conditions (1- to 3-s switch time) were selected to achieve compression of HMW DNA of >300 kb in order to visualize faint amounts of non-formamide-treated DNA.

formamide treatment and cloned this purified DNA into a BAC vector, the number of transformants obtained exceeded 80,000 clones, compared to only 19,000 BAC clones generated in 21 library attempts without using formamide treatment. Furthermore, fosmid cloning was also attempted with non-formamide-treated DNA, which did not result in any clones. In this study, there was not a concerted effort to achieve the largest insert sizes possible, since the primary concern was to first increase cloning efficiency. Presumably the increase in cloning efficiency achieved through formamide treatment would also enable more consistent larger-insert cloning; indeed, in a separate study (without negative controls that lacked formamide treatment), a soil metagenomic BAC library with

an average insert size of 68 kb has been constructed (data not shown). Taken together, these results indicate that an increase in the molecular weight of metagenomic DNA can be achieved through recovery of microbial cells from soil (i.e., indirect extraction) and that contaminants that coisolate with this metagenomic DNA from some soils may be removed by treatment with a formamide and high-salt solution within an agarose plug, resulting in an increase in the efficiency of metagenomic library construction.

TABLE 1. Soil metagenomic libraries constructed with or without formamide treatment

Library name	Library type	Nycodenz isolation ^a	Formamide treatment	No. of clones	Approx % insert	Avg insert size (kb)
AK 5	BAC	Yes	No	2,200	70	33
AK 7	BAC	Yes	No	2,300	60	47
AK 8	BAC	Yes	No	2,700	70	9
AK 9	BAC	Yes	No	2,400	25	20
AK 13	BAC	No	No	8,400	10	29
AK 17	BAC	No	No	1,000	20	29
AK 15	BAC	No	Yes	32,100	60	20
AK 9	Fosmid	No	Yes	3,300	98	31
AK 20	BAC	No	Yes	48,100	80	8
AK 21	Fosmid	No	Yes	333,700	100	30
AK 22	Fosmid	No	Yes	50,500	100	30
AK 23	Fosmid	No	Yes	8,200	100	30

^a In some cases, microbial cells were isolated over a Nycodenz layer as previously described (22).

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