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ORIGINAL ARTICLE

Isolation of High Molecular Weight DNA from Marine Sponge Bacteria for BAC Library Construction

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Abstract Metagenomics is a powerful tool for mining the genetic repositories from environmental microorganisms. Bacteria associated with marine sponges (phylum Porifera) are rich sources of biologically active natural products. However, to date, few compounds are discovered from the sponge metagenomic libraries, and the main reason might be the difficulties in recovery of high molecular weight (HMW) DNA from sponge symbionts to construct large insert libraries. Here, we describe a method to recover HMW bacterial DNA from diverse sponges with high quality for bacterial artificial chromosome (BAC) library construction. Microorganisms concentrated from sponges by differential centrifugation were embedded in agarose plugs to lyse out the HMW DNA for recovery. DNA fragments over 436 kb size were recovered from three different types of sponges, *Halichondria* sp., *Haliclona* sp., and *Xestospongia* sp. To evaluate the recovered DNA quality, the diversity of bacterial DNA comprised in the HMW DNA derived from sponge *Halichondria* sp. was analyzed, and this HMW DNA sample was also cloned into a shuttle BAC vector between *Escherichia coli* and *Streptomyces* sp. The results showed that more than five types of bacterial DNA, i.e., Proteobacteria, Nitrospirae, Cyanobacteria, Planctomycetes, and unidentified bacteria, had been recovered by this method, and an average 100 kb size insert DNA in a constructed BAC library demonstrated that the recovered HMW DNA is suitable for metagenomic library construction.

Keywords DNA recovery · Marine sponge · Bacterial artificial chromosome · Metagenomic library

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Introduction

Marine invertebrates especially sponges (phylum Porifera) are noteworthy for their unmatched diversity of secondary metabolites with promising potentials to become effective drugs for therapeutic applications (Sipkema et al. 2005). However, in the majority of cases, production of compounds derived from sponges (often of microbial origins) is impeded by inherent difficulties in collecting or culturing large quantities of these sponges (Kennedy et al. 2007), although sponge-derived compounds have pronounced anticancer (Paleari et al. 2006), anti-infective (Rao et al. 2004), or other bioactivities (Chang et al. 2003), making them interesting lead compounds for medical and biotechnological applications.

Sponges, as one of the oldest filter-feeder animals, absorb nutrients and remove microorganisms from seawater

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by pumping many thousands of liters of seawater per day through their aquiferous system (Li et al. 1998). In the process, most bacteria are eventually ingested by sponge archaeocytes. Some, however, survive in the mesohyl tissue and can be established as part of the sponge-specific microbiota (Kennedy et al. 2007). In certain demosponges, bacterial population may reach 10^8–10^{10} cells per gram of sponge in wet weight (exceeding seawater concentrations by 2–4 orders of magnitude), and the symbiotic bacteria contribute up to 40–60% of the sponge biomass (Kennedy et al. 2007). It is widely believed that many of sponge products are in fact produced by their bacterial symbionts (Newman and Hill 2006). For instance, diketopiperazines of the sponge *Tedania ignis* are ascribed to metabolites of *Micrococcus* sp. associated with the sponge (Stierle et al. 1988).

Therefore, the challenge in developing sponge-based bioactive natural product is to advance culturing and culture-independent approaches to exploit the rich genetic resources that sponge-microbial consortia offer and to allow the full assessment of their biosynthetic potentials. Despite progress being made in culture-dependent techniques for isolation of some sponge-associated microbes (Webster et al. 2001; Jiang et al. 2008; Zhang et al. 2008), the vast majority of sponge symbionts elude culture in the laboratory. Culture-independent metagenomic approach, providing valuable insights into the true origins of sponge-derived secondary metabolites (Piel et al. 2004a; Schirmer et al. 2005; Kim and Fuerst 2006; Fieseler et al. 2007), has opened up a new era that enables direct access to the genomes of numerous uncultivable microorganisms (Li and Qin 2005).

Metagenomics is proven to be a powerful tool for mining the diverse environmental microorganism resources (Rondon et al. 2000; Brady et al. 2001; Gillespie et al. 2002; Handelsman 2004; Venter et al. 2004). Currently, there are two approaches in research on metagenomics. In the first approach, massive sequencing facility was employed to decode metagenomic library with short sequences of fragments for assembly (Venter et al. 2004). In the second approach, scientists focus on potential of functional expression of metagenomic library clones with large insert DNA (Gillespie et al. 2002). In the later case, it starts with isolating DNA from an environmental sample, followed by cloning the DNA into a suitable vector, then transforming the clones into a host bacterium, and finally screening the resulting transformants (Handelsman 2004). In regard to the choice of a cloning vector, bacterial artificial chromosome (BAC) and fosmid (F1 origin-based cosmid vector) have both been successfully used in marine sample metagenomes (Beja 2004). Compared with fosmid libraries being capable of only screening up to 40 kb inserts, environmental BAC libraries enable the screening of larger DNA inserts up to 200 kb (Beja 2004), which can facilitate the reconstruction- and species-function analyses of environmental genomes (de la Torre et al. 2003) but demand larger DNA in size as the starting material. The most important strength of BAC vector in constructing an environmental DNA library, especially for drug development, is that a complete polyketide gene cluster could be embedded in a single clone to produce "unnatural natural product" compounds (Davies J and Li X, personal communication), since the size of some known biosynthetic gene clusters of metabolites exceed 40 kb, such as 55 kb of erythromycin (Brikun et al. 2004), 90 kb of avermectin (Ikeda et al. 1999), 107 kb of rapamycin (Schwecke et al. 1995), and 128 kb of daptomycin (Penn et al. 2006). Thereby, metagenomic libraries with average about 100 kb inserts will promote the natural product discovery, while some nature products were discovered from soil metagenome (Wang et al. 2000; Gillespie et al. 2002). However, there are few reports about nature products from sponge metagenomes or large insert metagenomic libraries of marine sponges. The main obstacle in this regard is perhaps the difficulty of efficiently recovering enough high quality DNA from sponge symbionts. In this paper, we describe a method for high molecular weight (HMW) DNA recovery from diverse sponge symbionts, and the quality of the recovered DNA is certified by a BAC library construction, and the diversity of the bacterial representatives comprised in the DNA sample of sponge *Halichondria* sp.

Material and Methods

Sponge Collection and Enrichment of Sponge Bacteria

Marine sponges *Halichondria* sp. and *Haliclona* sp. were collected at 2 m depth from Shantou (23°02′N, 117°06′E), and *Xestospongia* sp. at 10 m depth from Sanya (18°13′N, 109°29′E), China. The sponges identified by Chen W. Z. of Shantou University in China were stored at 4°C until they were used within 3 weeks. Fifty grams sponges were diced into small pieces and mechanically homogenized into cell suspension by a juicer in a 4°C TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). The cell suspension was first filtered by nylon mesh (50-μm pore size) and centrifuged at 250 g for 1 min to get rid of the sponge debris and dirt. Microscopic examination indicated few unicellular bacteria among the discarded pellet. The suspension was then centrifuged at 8,000 g for 20 min to concentrate the bacteria. The bacterial pellets were washed by TE100 (10 mM Tris–HCl, 100 mM EDTA, pH 8.0) until the sponge pigment was removed. The strategy here could concentrate both symbiotic bacteria and those simply adherent.

DNA Isolation

The genomic DNA of the enriched bacteria of *Halichondria* sp. was performed as described by the manufacturer's instructions (Kim and Fuerst 2006) by Genomic-tips 20/G (Qiagen, Germany), and the sodium dodecyl sulfate (SDS) lysis protocol for recovering the sponge bacterial DNA of *Halichondria* sp. described by Schirmer et al. (2005) was modified as follows: 0.5 g cell pellets were resuspended in 2 ml buffer (0.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 8.0) and treated with lysozyme (150 g/ml for 1 h at 37°C) and proteinase K (0.5 mg/ml), 1% SDS for 2 h at 50°C. After lysis, DNA was extracted with phenol-chloroform (two to three times), and chloroform and was precipitated with isopropanol.

The gel-embedding method developed here to recover HMW DNA from sponge bacteria was performed as follows: The cell pellets obtained from 50 g sponges were resuspended in 2.5 ml TE100 solution and were embedded in agarose plug by mixing with equal volume of 1.5% agarose solution. The fixed cells in agarose plugs were lysed in solution I (10 mM Tris–HCl, 50 mM EDTA, 1 mg/ml lysozyme, 0.1% 2-mercaptoethanol) at 37°C for 2 h. Then, the agaroses plugs were transferred to solution II (10 mM Tris–HCl, 250 mM EDTA, and 1 mg/ml proteinase K, 1% N-lauroylsarcosine, 0.1% 2-mercaptoethanol, pH 8.0) and incubated at 55°C for 1 h. These plugs were washed extensively with TE buffer, followed by inactivation of proteinase K with phenylmethylsulphonyl fluoride at 37°C for 1 h. Finally, the plugs were electrophoresed in clamped homogeneous electrical field (CHEF) apparatus (Bio-Rad) using 1.0% agarose in 0.5×Tris/borate/EDTA (TBE) buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3) at 14°C and 6 V/cm for 16 h with 0.1–40 s pulse time at a 120° angle. The over 400-kb size DNA band was recovered by cutting the corresponding agarose slices proportional to the quantity of the gel sample visualized by ethidium bromide staining as described before (Osoegawa et al. 1998). The recovered DNA was purified by electrophoresis again with the same setting described above to remove remaining small molecular DNA contaminants, and the HMW DNA gel slices were stored in 0.5 M EDTA (pH 8.0) at 4°C for BAC library construction.

Bacterial Diversity in HMW DNA of Sponge *Halichondria* sp. by 16S rDNA Survey

A 16S rDNA clones library was constructed using the HMW DNA of sponge *Halichondria* sp. symbiont recovered by gel-embedding method, with universal primers 27f: 5′-GAGTTTGATCCTGGCTCAG-3′ and 1500r: 5′-AGAAAGGAGGTGATCCAGCC-3′ (Woese et al. 1983). The amplicon of 27f/1500r were cloned to pMD18-T vector as the manufacturer's instructions (TaKaRa, China). Sixty-five positive clones were randomly selected for 16S rDNA restriction fragment length polymorphism (RFLP) analysis. The insert fragments were amplified by universal primers M13R-28 and M13F-20 from each clone and digested by restriction enzymes *Taq*I (TaKaRa, China). The digested DNA samples were electrophoresed on 2% agarose gel to show their RFLP patterns. The representative clones of each RFLP pattern were sequenced by using universal primers M13R-28 and M13F-20 by Invitrogen (Shanghai, China). Results were compared with known sequences in the GenBank database using the BLASTN search program (http://www.ncbi.nlm.nih.gov/) to determine approximate phylogenetic affiliation. The sequencing results deposited at GenBank, together with selected published sequences, were used for phylogenetic and molecular evolutionary analyses by MEGA version 4 (Tamura et al. 2007) with the neighbor-joining method (Saitou and Nei 1987).

BAC Library Construction: BAC Vector Preparation, Ligation, and Transformation

The BAC library construction approach was performed as Osoegawa et al. (1998) described. The vector pBAC-1003 is a 16-kb size shuttle BAC vector between *Escherichia coli* and *Streptomyces* sp., with pUC19 backbone (between the two *Bam*H I sites of the vector) and the apramycin resistant gene. Vector DNA, purified by PEG8000, was digested with *Bam*H I and treated with calf intestine alkaline phosphatase (TaKaRa, China) at 37°C. Dephosphorylated vector DNA was purified by electrophoresis to remove the pUC19 fragment. The linear vector DNA was ready for BAC library construction after electroeluting recovery.

HMW DNA isolated from *Halichondria* sp. was partially digested by *Mbo*I, and the treated DNA in 150–250 kb size was ligated to 50 ng prepared vector at an approximately 10:1 molar ratio. Ligation was performed at 16°C for 12 h. After the ligation, mixture was dialyzed with deionized water and concentrated by 30% PEG8000; 2 μl of the ligation mixture was used to transform 20 μl *E. coli* DH10B competent cells by electroporation. Cells were incubated to express an antibiotic resistance gene by shaking at 200 rpm for 1 h at 37°C in 900 μl of lysogeny broth (LB) medium. Then, cells were plated onto LB agar plates containing 5% sucrose and 75 μg/ml apramycin. The presence of *sacB* gene at the cloning site serves as a lethal factor for *E. coli* with empty vector.

Analysis of BAC DNA Library Clones

The 11 positive clones were randomly selected to extract the BAC DNA and digested with *Not*I restriction

enzyme (TaKaRa, China). The digested DNA was analyzed with CHEF apparatus on a 1% agarose gel in 0.5× TBE buffer at 14°C for 13 h and 5 V/cm with 5–15 s pulse time.

The mixture of BAC DNA extracted from the 11 clones was amplified by the universal primer set 27f/1500r. The amplicon was cloned to pMD18-T vector followed by 16S rDNA-RFLP analysis, and the representative clones of each RFLP pattern were analyzed by sequencing as described above.

Results

Sponge Bacterial DNA Isolation

To extract bacterial DNA from sponge, the total sponge tissue was homogenized into a cell suspension. The microbes were concentrated by 50-μm pore filtration followed by differential centrifugation to remove sponge debris and dirt from the microbial cell suspension. To determine the effect of the centrifugation, we used *E. coli* DH5α as a control to test at different speed. Under the conditions of low-speed centrifugation (250 g, 1 min), there is no significant pellet of *E. coli* found on the centrifuge tube compared with that at 500 g for 6 min, while sponge debris and dirt can be efficiently removed under this centrifugal conditions, and few unicellular bacteria were found in discarded precipitate.

Three different methods, i.e., Genomic-tips, SDS lysis protocol, and gel-embedding method, were used to recover the DNA of the concentrated microbes of sponge *Halichondria* sp. The DNA recovered by SDS lysis protocol is smeared mainly in 48.5–196 kb size, while the DNA samples isolated by Genomic-tips and gel-embedding method both form a strong DNA band of about 48.5 and over 436 kb size, respectively (Fig. 1c, d). Although all the DNA samples recovered by these three methods could be used to construct fosmid library, only the DNA recovered by gel-embedding is large enough in size to construct BAC library with average 100 kb insert. The DNA band over 436 kb size was recovered by cutting the corresponding agarose slices, followed with further electrophoresis purification, and the recovered HMW DNA was used for BAC library construction.

The gel-embedding method described here can be efficiently used to recover HMW DNA from different sponges. As the results, over 436-kb size DNA of mainly microbial origin was recovered from all three different sponges tested (Fig. 1a–c). The quantities of purified HMW DNA were 40, 24, and 25 μg per gram sponge, respectively, from *Halichondria* sp., *Haliclona* sp., and *Xestospongia* sp.

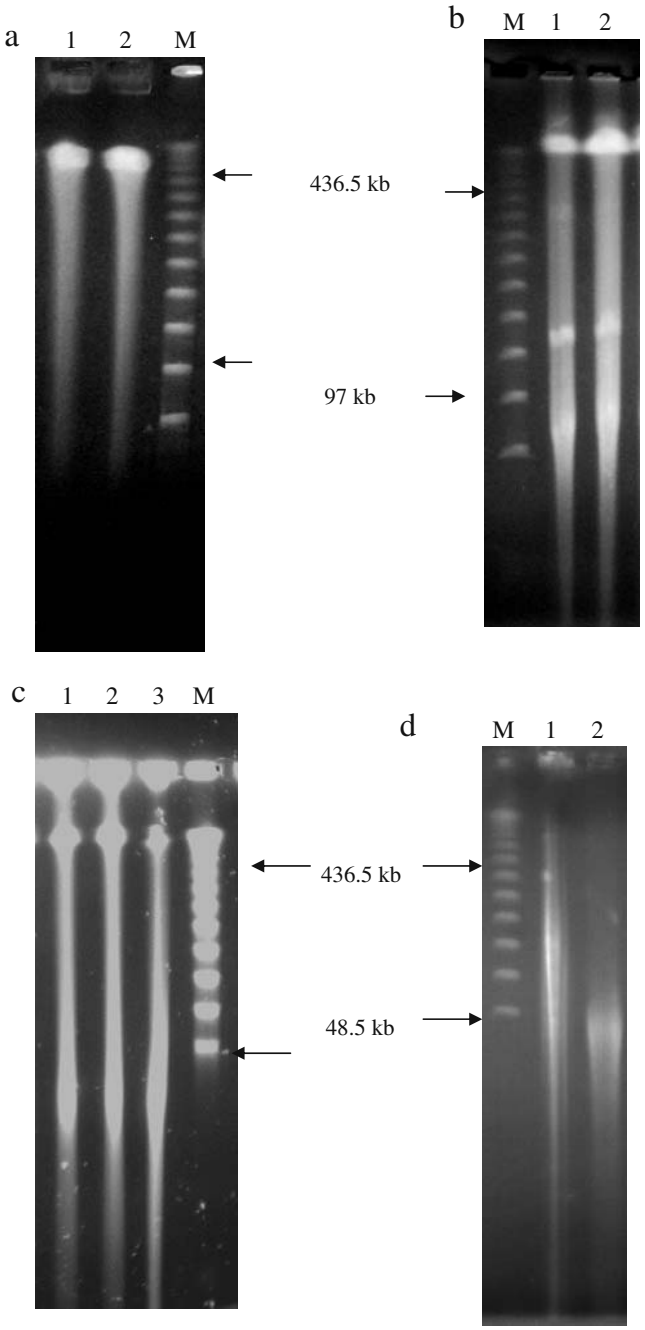

Fig. 1 Pulsed-field gel electrophoresis profile of sponge bacterial DNA. **a**–**c** DNA recovered by gel-embedding method. **a** Lane *1*, *2*: *Halichondria* sp. **b** Lane *1*, *2*: *Haliclona s*p. **c** Lane *1*–*3*: *Xestospongia* sp. *M*: lambda pulsed-field gel electrophoresis marker. **d** Lane *1*: DNA recovered by sodium dodecyl sulfate lysis method from *Halichondria* sp.; *2*: DNA recovered by Genomic-tips 20/G (Qiagen) from *Halichondria* sp.

Bacterial Diversity in HMW DNA of Sponge *Halichondria* sp.

To evaluate the quality and bacterial diversity of the DNA samples isolated by the gel-embedding method, we selected the DNA of sponge *Halichondria* sp. for a population analysis based on the 16S rRNA gene sequences. Sixty-five

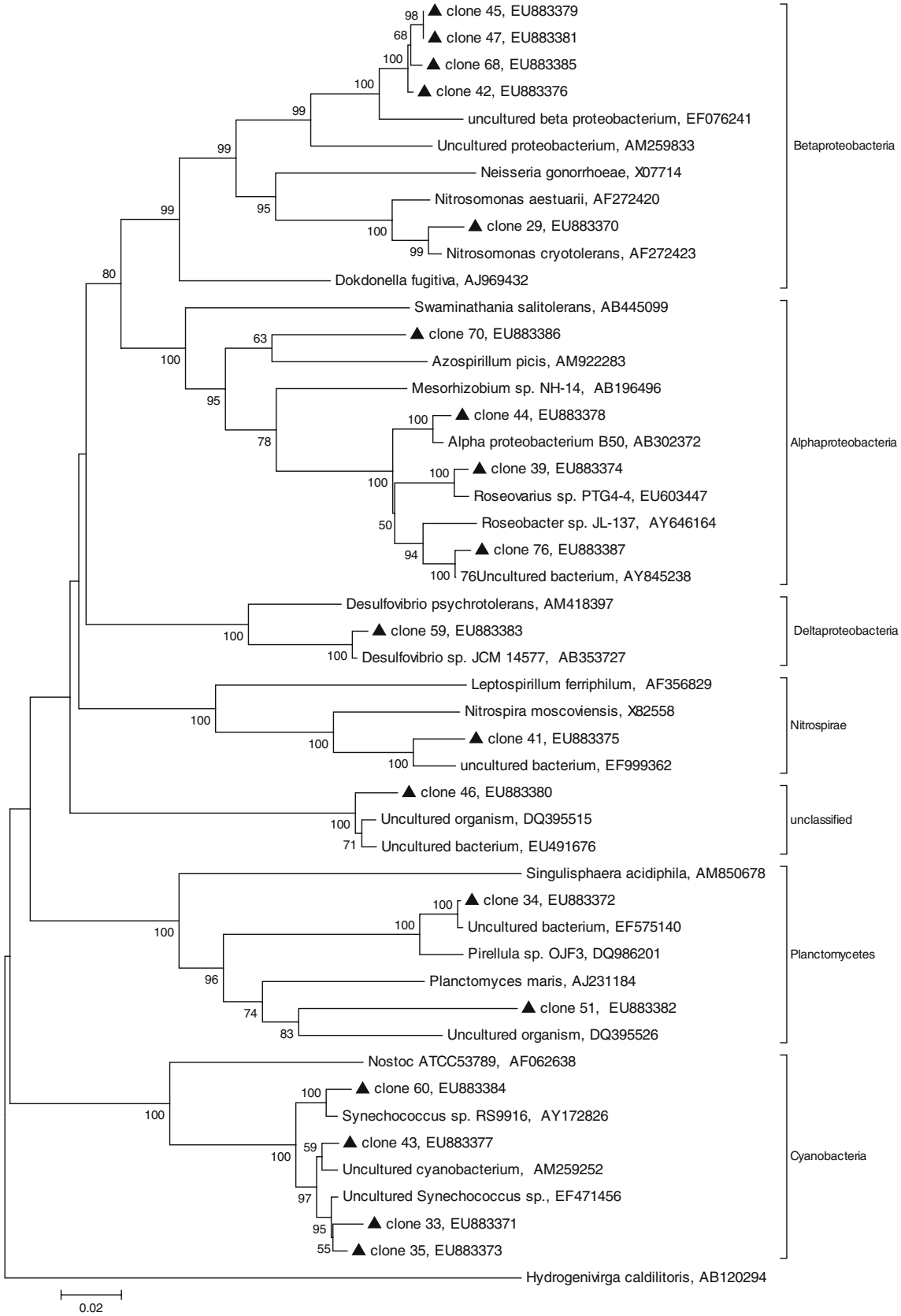

Fig. 2 Neighbor-joining tree of the 16S rDNA. The sequences of the branch marked by a *triangle* are from the sponge *Halichondria* sp. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater

randomly selected clones in a 16S rDNA library were analyzed by 16S rDNA-RFLP. The 65 amplified sequences showed 18 RFLP groups (data not shown) and each representative of RFLP pattern was followed by sequencing analysis. The result shows that the HMW DNA of the sponge *Halichondria* is rich in bacterial diversity (Fig. 2). The dominant phylum is Proteobacteria (63.0%, 41/65), and other bacteria include Nitrospirae (3%, 2/65), Cyanobacteria (27.7%, 18/65), and Planctomycetes (4.6%, 3/65). One clone (1.5%, 1/65) is cataloged as unidentified (Fig. 2).

The 16S rRNA gene sequences derived from sponge *Halichondria* sp. were assigned GenBank accession numbers EU883370–EU883387.

Analysis of BAC DNA

The desalted ligation mix containing prepared vector pBAC-1003 and sponge *Halichondria* sp. derived 150–250 kb size partially digested DNA was transformed to DH10B competent cells by electroporation. In a small scale transformation, 518 clones were obtained from the LB agar plates containing sucrose and apramycin. Eleven random clones were selected to analyze the inserted DNA size. The pulsed-field gel electrophoresis showed that the average size of the inserted DNA is about 100 kb (Fig. 3). The amplicon of 16S rDNA sequences from the mixture of 11 BAC clone DNA was further cloned to *E. coli* for RFLP analysis. Over 215 analyzed sequences derived from the BAC DNA showed two types of RFLP patterns (data not shown), and each representative of RFLP patterns was sequenced. The sequencing results indicated that the sequence of one pattern, which shared 99% identity with 16S ribosomal RNA gene of *E. coli* (CP000948; data not shown), may be amplified from the host strain *E. coli* DH10B, while the sequence of the other pattern, deposited in GenBank with accession numbers FJ847941, should be originated from the gamma proteobacterium associated with sponge *Halichondria* sp.

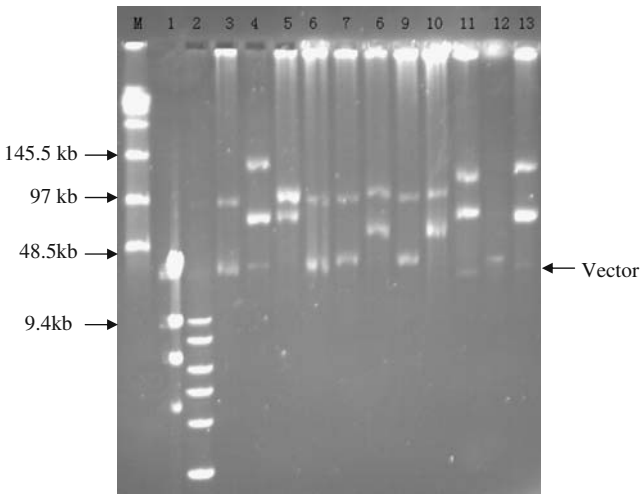

Fig. 3 Pulsed-field gel electrophoresis analysis of BAC DNA derived from the sponge *Halichondria* sp. The extracted BAC DNA digested with *Not*I restriction enzyme and analyzed in a 1% agarose gel in 0.5× TBE buffer at 14°C for 13 h and 5 V/cm with 5–15 pulse time. Lane *1*: lambda/*Hind* III marker; *2*:1–11 kb DNA ladder, 3–13 represent 1–11 randomly selected BAC clone, respectively; *M*: lambda pulsed-field gel electrophoresis marker

Discussion

Metagenomic BAC library is ideally suited for genetic characterization of uncultured bacteria associated with sponges and screening for gene fragments or gene clusters from biosynthetic pathways involved in the production of secondary metabolites (Kennedy et al. 2007; Piel et al. 2004b; Schirmer et al. 2005; Kim and Fuerst 2006; Fieseler et al. 2007). Moreover, the bioactive mentalities' genes which were discovered by metagenomic approach, such as polyketide synthase (PKS) genes, were not shown in cultural bacteria, and these PKS from sponge metagenomes formed a marine sponge-specific PKS cluster (Kim and Fuerst 2006). With regards to the specific sponge metagenomic libraries, different methods have been employed to isolate the HMW DNA of symbionts, and the composition of the sponge metagenomic libraries are relatively dependent on the procedures used in DNA recovery. As Schirmer et al. (2005) reported, the metagenomic libraries of different types of sponge bacteria separated by different centrifugation speed were characterized by different G+C, PKS, and nonribosomal peptide synthetase (NRPS) profiles. Especially, the metagenomic library made from filamentous bacteria concentrated by low centrifugation speed (500 g, 6 min) was notably different from existing libraries for containing numerous NRPS and mixed NRPS-PKS gene clusters (Schirmer et al. 2005). In order to get the most representatives of the sponge-microbial community for BAC metagenomic library construction, we have been careful to discard the sponge debris and dirt from the sponge suspensions by differential centrifugation. This is equivalent to the conditions of the slow speed and the short duration centrifugation protocol (i.e., 250 g, 1 min), in which most of the dirt and sponge debris constitute the pellets, and few unicellular bacteria are found in the discard pellets. It seems that this protocol introduces little bias on the physical separation of the sponge microbes. The concentrated cells will most closely represent microorganism community associated with the sponges.

Environmental BAC libraries enable heterogenous expressing of larger inserts when compared to fosmid libraries, thus facilitating natural product discovery. However, sufficient extraction of HMW DNA from sponges is

the major bottleneck in BAC metagenomic application, although several fosmid libraries of sponge metagenomes have been used to mine the sponge bacterial metabolites, e.g., onnamide (Piel et al. 2004b). In this paper, we developed a method to recover HMW DNA from sponge microbes for BAC library construction, and the recovered DNA is over 436 kb, more larger in size than that of DNA isolated by other methods described before, e.g., Genomic-tips 20/G (Kim and Fuerst 2006) and SDS lysis (Schirmer et al. 2005; Fig. 1c, d). To evaluate the quality of HMW DNA recovered by gel-embedding method, we demonstrated a BAC library construction with a shuffle vector which facilitates the insert DNA to express in streptomycetes. The BAC DNA analysis showed that the average size of the insert DNA in the small scale BAC library is about 100 kb, a fragment size that generally required for expression of an intact gene cluster in a surrogate host. Analysis of the 16S rDNA-RFLP and sequencing indicated that the inserts of the clones are derived from sponge bacteria. In addition, the DNA recovery approach described here does not need a centrifugal medium (e.g., Nycoden) for cell separation when compared to other DNA recovery methods for construction of large insert BAC libraries from different environments (Berry et al. 2003; Bertrand et al. 2005). This approach could apply to diverse sponges since similar size HMW DNA was recovered from three different types of sponges (Fig. 1a–c).

The diversity of bacteria DNA in the HMW DNA recovered by gel-embedding method from sponge *Halichondria* sp. has been evaluated by 16S rRNA gene. The HMW DNA of sponge *Halichondria* sp. consists of more than five types of bacterial DNA, i.e., Proteobacteria, Nitrospirae, Cyanobacteria, Planctomycetes, and unidentified bacteria (Fig. 2). As described in other studies of sponges from the South China Sea (Li et al. 2006), the phylum Proteobacteria are the most predominant sponge-associated bacteria. With equal importance, the Proteobacteria have shown multilateral effects on the hosts, such as nitrogen fixation (Burnett and McKenzie 1997), sulfate-reducing function (Hayes and Lovley 2002), producing antimicrobial and surface-active compounds (Kalinovskaya et al. 2004), and enzymes of degrading polysaccharides (Groudieva et al. 2004). Another dominant group of bacteria concentrated in this study is the Cyanobacteria. These bacteria are considered to be the true biogenic source of a number of pharmacologically active polyketides and nonribosomal peptides found in the sponges (Dunlap et al. 2007). A particularly exciting discovery from Cyanobacteria is cyanovirin. It has been found to be active against immunodeficiency retroviruses HIV-1, HIV-2, SIV (simian), and FIV (feline; Boyd et al. 1997) and has high potency against most strains of influenza A and B viruses (O'Keefe et al. 2003). It follows logically that these HMW DNA containing the above discussed uncultured microbial genetic resources represent a gold mine for drug discovery. It is believed that rapid progresses in metagenomics, e.g., large insert libraries construction and multi-host expression of heterogenous gene clusters, would make efficient way for exploiting the sponges' uncultured bacteria.

In summary, the method we have described here for HMW DNA recovery from sponges and cloning into a shuttle BAC vector provides a viable strategy for surrogate host expression of metagenomic BAC library. This could open the door to drug discovery from marine sponges harboring a treasure trove of a wide range of unculturable microorganisms.

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