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Note

An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities

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Abstract

We propose a strategy for the efficient screening of large libraries of amplified 16S rRNA genes from complex environmental samples. It consists of processing sets of multiple clones simultaneously. This strategy saves up to 90% of the costs and labor spent in the process of screening a 16S rDNA library.

Keywords: Fingerprinting methods; Screening; Microbial diversity; DGGE; Cloning; 16S rDNA

Trends in current microbiological research are repeatedly highlighting the interest of microbial diversity to understand the functioning and structure of complex microbial communities (Ward et al., 1990; Massana et al., 1997; Pace, 1997; Vetriani et al., 1998). Since the majority of microorganisms in most environments cannot be cultured following standard procedures (Roszak and Colwell, 1987; Ward et al., 1990; Hugenholtz et al., 1998; Dunbar et al., 1999), molecular techniques are essential tools for analyzing microbial diversity. Today, molecular fingerprinting techniques are commonly used for detecting microbial diversity in natural samples. Among a number of available fingerprinting techniques, denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Massana et al., 1997; Diez et al., 2001; Jackson et al., 2001) and terminal restriction fragment length polymorphisms (t-RFLP) (Liu et al., 1997; Braker et al., 2001; Dunbar et al., 1999) are frequently used. Both techniques require the amplification of specific DNA sequences, generally by PCR, and the use of electrophoretic methods. The most frequently targetted DNA fragment in prokaryotic diversity studies is the 16S rRNA gene. By comparing community fingerprints obtained from different experimental treatments, conditions, or at different time points, one can select characteristic bands to the process under study. However, these bands need to be identified as belonging to either a specific microbial species or a novel microbial group. The general procedure recommends the construction of a 16S rDNA library by cloning the amplified DNA fragments into a host vector and transformation in *Escherichia coli* (Sambrook et al.,

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1989; Ward et al., 1990; Massana et al., 1997; Pace, 1997; Vetriani et al., 1998; Zwart et al., 1998; Dunbar et al., 1999; Orphan et al., 2001). Processing a DNA library requires the screening of its clones and the sequencing of the selected clones. 16S rDNA library screening is a costly, time-consuming process. Since ribosomal RNA genes are highly conserved between microbial species, recently developed DNA substraction techniques (Bjourson et al., 1992; Buchaille et al., 2000) do not work properly. Screening by labelled oligomer probes cannot be used since a priori the sequence to be targeted is unknown. The standard screening procedure consists in examining a number of clones using molecular fingerprinting techniques (e.g., DGGE analysis) until the clone carrying the DNA fragment of interest is found. When the clone of interest represents a minority in a 16S rDNA library, this procedure often misses the desired clone due to the difficulty, both in timing and costs, of processing a large enough set of clones (Hughes et al., 2001). Herein, we propose an interesting strategy for performing efficient, low cost, low time-consuming, screenings especially useful for rDNA libraries or highly similar DNA sequences.

Using molecular fingerprinting techniques (i.e., DGGE) complex microbial communities often result in the visualization from tens up to hundreds of different amplified rDNA sequences (Hughes et al., 2001). In order to identify the microbial species corresponding to specific bands of interest, there are two possible alternatives. The first possibility would be to excise the band of interest (i.e., from a DGGE gel), re-amplify and sequence that DNA fragment (Gich et al., 2001). DGGE fingerprinting analysis should be performed with DNA fragments shorter than 400 nucleotides (Muyzer et al., 1996); generally, DNA fragments of about 200 bases are amplified for DGGE analysis on 16S rRNA genes (Muyzer et al., 1996). These short DNA fragments provide very limited phylogenetic information. Another inconvenient of this alternative is the high probability of picking multiple DNA sequences while cutting off the band of interest from a DGGE gel; this would require additional cloning and screening for obtaining single 16S rDNA sequences. The second alternative consists in cloning the amplified DNA fragments by constructing a rDNA library. This rDNA library will undergo a screening process aimed to selecting those clones of interest. When working with complex communities, the number of different clones can be quite high resulting in a lengthy, costly, and time-consuming process (Hughes et al., 2001). However, a major advantage of this second alternative is that longer DNA sequences can be retrieved maximizing the phylogenetic information to be obtained. If the screening process can be shortened down to reasonably low cost and labor-consuming levels, microbial diversity analyses would significantly benefit and high number of clones could be processed.

Recently, we have encountered these problems when analyzing the microbial diversity of biofilms covering stainless steel coupons in a sewage treatment plant in Jerez de la Frontera (Cadiz, southwestern Spain). An elevated number of bands corresponding to 16S rDNA amplified sequences were detected by DGGE analysis (Fig. 1). We were interested in identifying 16S rDNA fragments corresponding to specific bands detected on the DGGE gel. Screening a 16S rDNA library for bands of interest would require intense labor and elevated costs falling out of the available budget. Bands of interest can be detected when comparing two DGGE fingerprints from differ-

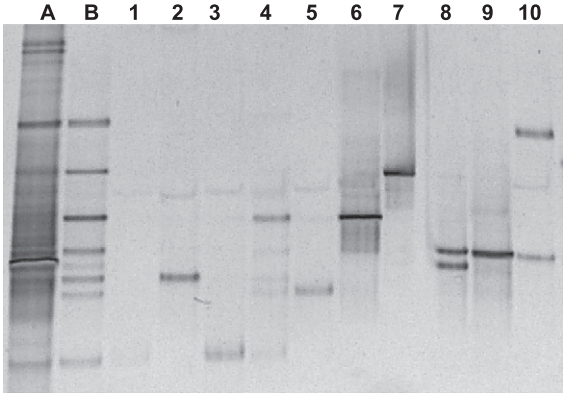

Fig. 1. DGGE analysis showing a representative example of the screening process outlined in this study. Lane A shows the amplification products of 16S rDNA fragments from a DNA template extracted from a biofilm. Lane B shows bands corresponding to the amplification of DNA fragments from a set of 10 clones of a 16S rDNA library prepared from the same DNA template used for lane A. Lanes 1 to 10 show the amplification products obtained from individual clones belonging to the clone set shown in lane B. The individual clones (lanes 1 to 10) can be easily identified in the set of 10 clones (lane B).

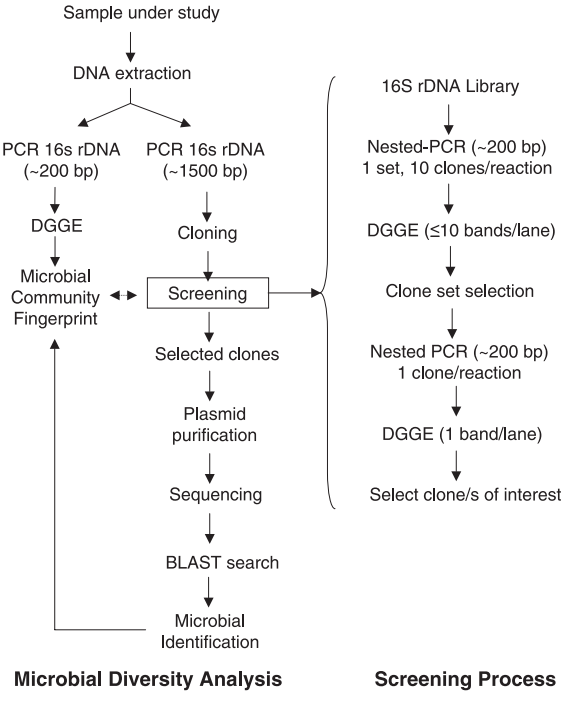

Fig. 2. Chart showing the procedure for microbial diversity analysis of environmental samples using DGGE and the proposed strategy for an efficient screening of 16S rDNA libraries.

ent samples or treatments. This type of differential analysis allows to identify bands specifically present in only one of the samples, or bands showing significant changes in their abundance when two samples or experimental treatments are compared. Certainly, this problem has been frequently encountered by numerous microbiologists during biodiversity studies.

DNA was extracted as previously described (Tsai and Olsen, 1991) from the samples mentioned above. 16S rDNA sequences were amplified by touchdown PCR (Massana et al., 1997). The primer pair used for this amplification were 341F (with a GC-rich tail) and 519R as suggested by Muyzer et al. (1993) for DGGE analysis of bacterial 16S rRNA genes. Microbial diversity was analyzed by obtaining a fingerprint of the community using DGGE (Fig. 1, lane A). DGGE was performed as described by Muyzer et al. (1996) using the primer pairs mentioned above. To identify representative bands visualized by DGGE, we followed basically the protocol described by Schabereiter-Gurtner et al. (2001) with modifications aimed to reduce the work load and the costs required by the screening procedure. Briefly, we amplified the 16S rDNA sequences of the DNA extracted from the environmental samples using the primer pair 27F and 1497R (Orphan et al., 2001). The PCR products were cloned using the TA-TOPO-cloning kit (Invitrogen). Clones from this rDNA library were selected by the following screening process. The clone insert was amplified by nested PCR using the plasmid-specific primer pair T7 promoter and M13 reverse as first primer pair and the primer pair used for DGGE analysis (see above) as the second primer pair. The unique aspect of the screening process is the DNA template used in this nested PCR. As DNA template we used a mixture of 10 clones. Each set of 10 clones was prepared by collecting cells from each of the individual clones. Cells from each clone were suspended in a common 200 μl TE buffer. This clone mix was lysed by three freeze/thaw cycles and 1 μl of a 1:1000 dilution was used as DNA template for the nested PCR. The obtained PCR products were analyzed by DGGE. A set of 10 clones containing the band of interest was selected for the second step of the screening process (Fig. 1, lane B). The 16S rDNA fragment cloned in each of these 10 clones of the selected set was amplified by the nested PCR described above but, in this case, the DNA template was composed by single clones. Cells from each single clone were suspended in 20 μl TE buffer. The resulting PCR products were analyzed by DGGE as above (Fig. 1, lanes 1 to 10). The clone carrying the band of

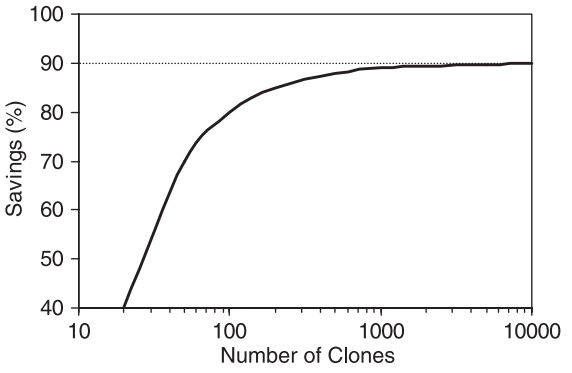

Fig. 3. Chart showing the reduction in cost and working labor when using the proposed screening strategy. Note the position of the asymptote (dotted line) to the curve at 90% savings.

interest can be identified by comparative DGGE analysis of these clones and the original amplification products (Fig. 1). Selected clones were sequenced using standard protocols at the Centre of Biological Research (CIB-CSIC, Madrid, Spain). The outlined protocol and proposed screening strategy are summarized in Fig. 2.

As an example, we can consider the mathematics for an experiment requiring to process 100 clones of a 16S rDNA library in search of a clone carrying an unknown 16S rDNA fragment corresponding to a desired band from DGGE analysis. Amplification of DNA inserts from 100 clones would require 100 PCR reactions and 100 lanes for DGGE analysis. Using the 10 clones/PCR reaction strategy proposed in this study, only 10 PCR reactions and 10 lanes for DGGE analysis are required to select a set of 10 clones; 10 additional PCR reactions and 10 lanes for DGGE analysis will be enough to localize the clone of interest within the selected set. In summary, the proposed strategy (Fig. 2) requires only 20 PCR reactions and 20 lanes for DGGE compared with the 100 reactions and DGGE lanes of the standard protocol. In our example, this strategy represents savings of 80% in costs and working labor. Although this example was based on the assumption of analyzing only 100 clones, the method can be scaled up and any large number of clones can be screened in a much shorter time frame than following standard methodology. In fact, most studies generally perform screenings of more than 100 clones (Hughes et al., 2001). Fig. 3 shows the cost/labor reduction for increasing numbers of sampled clones. The curve's asymptote is located at a level of 90% savings from analyses of around 1000 clones, and the proposed strategy can represent significant savings for screenings from 20 clones. Our screening strategy results in reducing costs, time, and working labor by up to 90% depending on the number of clones to be analyzed.

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