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Cloning Method for Taxonomic Interpretation of T-RFLP Patterns

Terminal RFLP (T-RFLP) is a recently introduced PCR-based tool for studying diversity among amplified DNA molecules with the same size (4). Terminal restriction fragment (TRF) patterns obtained using the T-RFLP technique are generated and analyzed in a procedure that combines PCR with a labeled primer, restriction digestion of the amplified DNA, and separation of fragments in an automated sequencer. One of the most interesting applications of T-RFLP is its use for the description of genetic diversity within a bacterial community (2,3,7). In this case, the PCR is performed using primers annealing to conserved sequences of 16S rRNA gene, and the TRF pattern obtained represents a "picture" of the number of different 16S rRNA genes present in the community (i.e., different operating taxonomic units). One of the main limits of this technique is the difficulty of obtaining taxonomic information of the organisms responsible for a particular TRF. To allow the interpretation of TRFs, an online tool has been developed called TAP (T-RFLP Analysis Program; Reference 5) that performs in silico PCR amplification and restriction of the 16S rDNA sequences present in the Ribosomal Database (RDPII, Center for Microbial Ecology at Michigan State University, http://rdp.cme.msu.edu/html/TAP-trflp.html#program). However, database matching of TRF sizes is imprecise and may not produce species- or even genus-specific assignment (2), and results can be experimentally verified indirectly only after a long screening of a 16S rDNA library.

Here we present a simple and fast method that allows for an empirical taxonomic interpretation of TRFs. This method would be particularly useful when a detailed taxonomic description of a bacterial community is needed. Figure 1 depicts the flow chart of the methodology. The method is based on the ligation of the TRFs with oligonucleotide adapters, subsequent PCR-amplification, cloning, and final sequencing of the single TRFs.

We used this technique to identify the bacterial groups represented in a TRF pattern obtained from soil DNA. DNA was extracted with the Fast Prep DNA Kit for Soil™ (BIO101, Qbiogene, Carlsbad, CA, USA) from 0.5 g fresh soil collected in a wood of cypress near Florence (Italy). The 16S rDNA was amplified in a 50-μL volume with 2 U *Taq* DNA polymerase (Dynazyme II™; Finnzyme, Espoo, Finland) using 27f primer labeled with TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein) and 1495r primer (6). The amplified products were purified with a QIAquick™ PCR purification kit (Qiagen, Valencia, CA, USA), and 600 ng were digested with 20 U *Msp*I or *Hha*I (New England Biolabs, Beverly, MA, USA) for 3 h at 37°C. The digested products (200 ng) were resolved by capillary electrophoresis on an ABI 310 Genetic Analyzer™ using TAMRA 500 as size standard for GenScan™ (all from Applied Biosystems, Foster City, CA, USA) analysis. The restricted products (200 ng) were ligated with 10 pmol complementary double-stranded adapter with 10 U T4 DNA ligase (New England Biolabs) for 10 h at 14°C in a 10-μL volume. One microliter of the ligation reaction was PCR-amplified using 27f
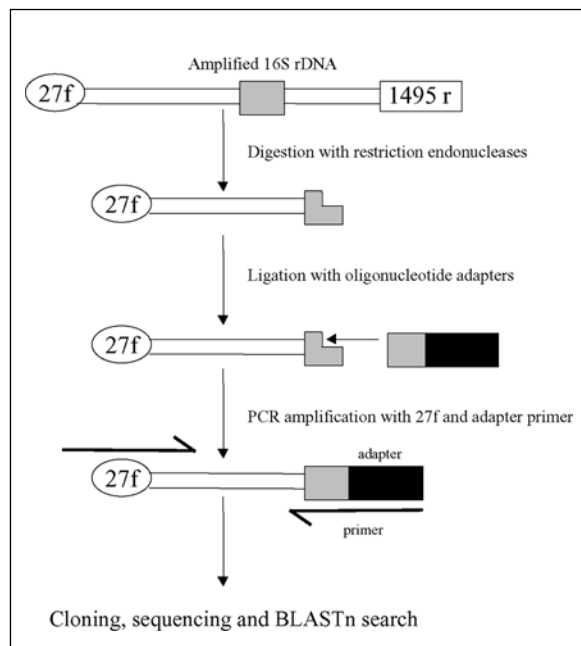

Figure 1. Schematic representation of the cloning system. The nucleotide sequences of the double-stranded adapters used are as follows. For *Msp*I, 5′-CGCTCGTAGACTGCGTACC-3′ and 3′-GAGCATCTGACGCATGGTTAA-5′. For *Hha*I, 5′-GAGCATCTGACG-CATGGTTAA-3′ and 3′-GCCTCGTAGACTGCGTACC-5′. The PCR amplification of the ligated products was performed using the following temperature profile in a Primus 96™ Thermal Cycler (MWG-AG-Biotech, Ebersberg, Germany): 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C.

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and pMspI (5′-GGTACGCAGTCTAC-GAGCGG-3′) or pHhaI (5′-CCATGC-GTCAGATGCTCCGC-3′) reverse primers, which were designed on the sequence of the double-stranded adapter and on the half restriction site of *Msp*I and *Hha*I, respectively. The amplified fragments were resolved on a 2% agarose gel, and the bands at molecular weights from 30 to 300 bp were eluted from the gel (MinElute™ Gel Extraction Kit; Qiagen) and cloned in the pDrive™ vector (Qiagen PCR Cloning Kit; Qiagen). This procedure allows for great reduction and faster screening of the clone library needed, when compared with cloning the original 16S rDNA products before digestion. A rapid screening for clones of appropriate size corresponding to specific TRF peaks was performed by PCR, using the standard M13 primer pair (M13-forward primer labeled with TET), and the size of the cloned TRFs was verified by capillary electrophoresis of the amplified products in the ABI 310 Genetic Analyzer. All cloned and sequenced fragments were represented in the T-RFLP profile. The most abundant fragments in the original T-RFLP were found to be predominant also in the library. However, a bias toward a higher content of fragments around 100 bp was found, probably due to PCR amplification. Twelve selected clones (six for each of the two enzymes) were then sequenced using the BigDye™ Terminator (v.2) chemistry (Applied Biosystems). Sequences were used to perform a BLASTn search (1) on the GenBank-EMBL-DDBJ database. All the obtained fragments were found to be significantly similar to sequences present in GenBank, with score and E-value varying from 44 and 1e-3 for the smallest fragment (33 bp) to 321 and 1e-85 for larger fragments (202 bp). In general, the longer the fragment, the higher the score is. The taxonomic interpretation derived from BLAST matching was then compared with that obtained by using the TAP tool; in four cases out of the 12 analyzed (TRFs produced with *Msp*I at 64, 202, 212, and 33 bp), TAP failed to show any matching, while the BLAST matching of the sequences found that those fragment were similar to 16S rDNA bacterial sequences present in GenBank (with score and E-values varying from 46 and 1e-3 to 321 and 1e-85). In two cases, the BLAST matching of the sequenced TRF was different from the matching produced by TAP (TRFs produced with *Msp*I at 174 bp and with *Hha*I at 38 bp). In the remaining cases, the two procedures were well in agreement (TRFs produced with *Msp*I at 68 and 145 bp and with *Hha*I at 82, 86, 89, and 95 bp). The reported results demonstrate that the direct cloning of TRFs could be a useful tool, complementary to TAP matching, for the taxonomic interpretation of T-RFLP community profiles.

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Molecular Cloning Using the Interrupted Recognition Sequence of *Bpl*I to Select for Recombinant Molecules

The cloning of DNA fragments into a uniquely restricted target vector is a common procedure for constructing genomic DNA or cDNA libraries. The recovery of recombinant molecules is