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eres after hundreds of cell divisions (6). This lengthening occurs only after a sufficient number of basal wild-type repeats have been replaced with mutant ones. For the Kpn telomere, 11 repeats could be altered using a single oligonucleotide, as was done with the totally Bcl telomere mutant. The partial repeat bordering the sub-telomere does not contain the positions being altered in the Kpn mutant but may retain a binding site for Rap1p, a negative regulator of telomere length (3). To disrupt Rap1p binding and remove the possible negative regulation conferred by the border repeat, we added an oligonucleotide that targeted a base change to the fourth nucleotide of the telomeric repeat adjacent to sub-telomere. If incorporated, this mutation would generate an *AccI* restriction site (Figure 1C). When templated by telomerase, this mutation causes telomere elongation (6). The procedure was followed as described above for the Bcl mutant, except that 6 ng kinased oligonucleotide specific to the most basal repeat was also used.

All four of the recovered plasmid clones contained novel *AccI* and *KpnI* sites, as indicated by restriction digests. These clones were then sequenced, and one containing all of the expected mutations was identified. The clones that were not totally mutated had most repeats containing the expected Kpn mutation, but they also contained from one to three wild-type repeats. It is possible that the failure to mutate all of the repeats was due to an insufficient amount of Kpn oligonucleotide in the reaction.

To introduce the totally Bcl telomere into *K. lactis*, an *EcoRI* + *SacII* digest was first done to remove the telomere and its associated *URA3*-tagged sub-telomeric sequence (Figure 1A). The digest was then transformed into a *ura3* *K. lactis* strain. Plates lacking uracil were then used to select for cells in which one native telomere was replaced through homologous recombination between the sub-telomeric sequences. Figure 2 shows restriction digests with *SmaI* and *BclII* that confirm the presence of the totally mutated Bcl telomere in the yeast. *SmaI* cleaves the *URA3*-containing telomere into a fragment, which then separates from the other telomeric fragments in the cell. As expected, *BclII* digestion cleaves away the *URA3*-Bcl

telomere but not a control *URA3*-wild-type telomere. In other experiments, by transforming the complete Bcl telomere into a *K. lactis* strain containing a mutant telomerase RNA gene (*TER1*) that adds Bcl repeats onto telomeric ends, we have made strains with one telomere completely composed of Bcl repeats (data not shown).

In conclusion, we have shown that oligonucleotide mutagenesis can be used to simultaneously alter every repeat in a tandem array of short repeats. This has allowed us to generate a totally mutant telomere in yeast. Although it is unclear if our approach can be used in systems with the 5–8 nucleotide repeats typical of most eukaryotes, it will be a valuable tool in systems such as *K. lactis*, which contain long uniform telomeric repeats. It could also be used for the mutagenesis of other kinds of repeated sequences. It is possible that by increasing the molar excess of mutagenic oligonucleotide, the procedure can be adapted to simultaneously mutate a higher number of repeats or smaller repeats.

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## Streamlined Method to Analyze 16S rRNA Gene Clone Libraries

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In the past decade, extraordinary progress has been made in the identification of bacterial species in natural communities, particularly because of phylogenetic comparisons of the 16S rRNA gene (1,4,9,10). Typically, this gene is amplified from a population of bacteria using the PCR, cloned into a commercial vector to create a library of 16S rRNA gene clones, and then various methods are used to screen and identify cloned inserts. Our laboratory has processed several such libraries; in this report, we describe a fast, cost-effective method to fully characterize this type of library. Many of the methods would also apply to clone libraries containing other gene inserts.

The streamlining of this method begins after 16S rRNA genes have been amplified and cloned into a suitable plasmid vector. We have had success using the pCR<sup>®</sup>II vector (Invitrogen, Carlsbad, CA, USA), and the pGEM<sup>®</sup>-T Easy vector (Promega, Madison, WI, USA). Both vectors contain M13 reverse and forward priming sites, which flank the inserted 16S rRNA gene.

Clones that have been streaked for isolation are grown overnight at 37°C

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in 3 mL LB broth (8) containing an antibiotic appropriate for the vector. Cultures are then aliquoted in the following amounts: 100  $\mu$ L into each well of a 96-well microplate, 1.5 mL to a microcentrifuge tube, and 1  $\mu$ L to a PCR tube. These three aliquots will be used to archive the clones, isolate the plasmid DNA, and generate a template for PCR and subsequent restriction fragment length polymorphism (RFLP) analyses (Figure 1).

After distributing the liquid culture for each clone to the 96-well microplate, 100  $\mu$ L 14% dimethyl sulfoxide (DMSO)/LB is added to give a final concentration of 7% DMSO. The plate is then sealed and stored at  $-80^{\circ}\text{C}$ , creating an archive of clones that can be re-streaked and regrown if necessary.

Cultures in the 1.5 mL microcentrifuge tubes are pelleted. The supernatant is discarded, and the pellets are stored at  $-20^{\circ}\text{C}$  until RFLP screening of all clones has been completed. For the clones that require sequencing, plasmids can be isolated from these pellets using the standard commercial mini-prep kits.

Clone library characterization starts with PCR amplification of the cloned 16S rRNA gene using the 1  $\mu$ L culture. Each 20  $\mu$ L PCR contains 1 $\times$  commercial *Taq* buffer, 2.5 mM Mg, 5% acetamide (7), 0.5 U *Taq* DNA polymerase (MBI Fermentas, Hanover, MD, USA), 200  $\mu$ M dNTP mixture, and 200 nM commercially available M13 forward and reverse primers. Cycling parameters for a PTC-100<sup>TM</sup> thermocycler (MJ Research, Watertown, MA, USA) are  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 2 min, repeated for 35 cycles. No processing of the *E. coli* culture is required before the PCR analysis because the first few cycles of the PCR are sufficient to break open the cells and make plasmids available for amplification and because the *E. coli* cells do not appear to inhibit the PCR. M13 primers are used to ensure that the entire insert is amplified. The use of 16S rRNA specific primers would also amplify the *E. coli* 16S rRNA gene, which would confound the analysis by generating unintelligible RFLP patterns and possibly by generating false positives.

PCR products (5  $\mu$ L) are electrophoresed on a standard 1% agarose gel. The product length may vary

**Table 1. Similarities Between Different Sets of RFLP Subgroups**

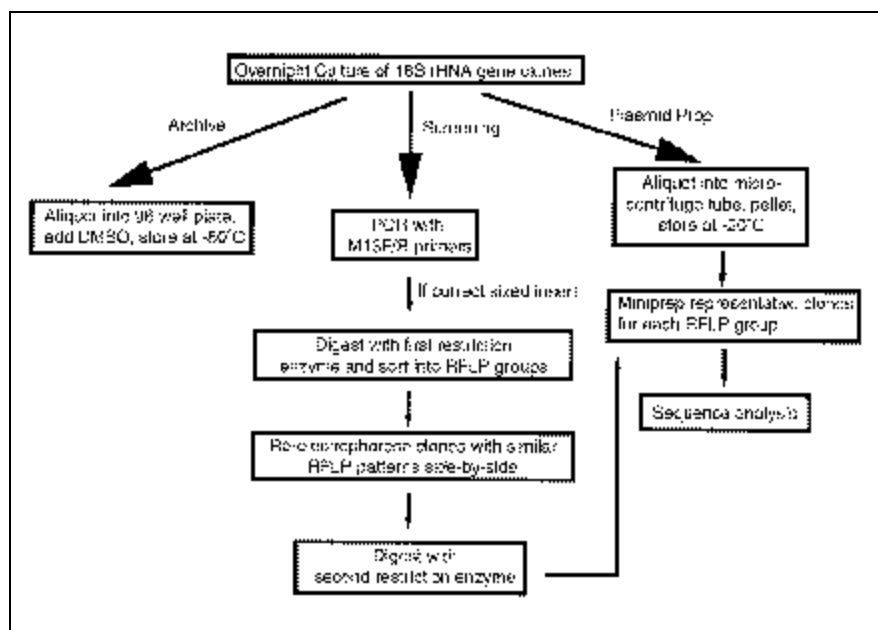
RFLP Subgroup	Number of Representatives	Similarity <sup>a</sup> (%)	
		Within Subgroup A	Compared to Subgroup A
IA <sup>b</sup>	3	99.7–100	
IB	1		99.7–100
IC	1		79.3–79.7
IIA <sup>c</sup>	4	98.7–100	
IIB	2		95.2–98.3
IIC	1		95.6–96.0
IIIA <sup>d</sup>	5	99.2–100	
IIIB	1		99.4–100
IIIC	1		99.8–99.9

<sup>a</sup>Sequences were aligned using the ARB software (5), and similarities were determined using PHYLIP (3).  
<sup>b</sup>Mask for group I is 285 nucleotides.  
<sup>c</sup>Mask for group II is 480 nucleotides.  
<sup>d</sup>Mask for group III is 502 nucleotides.

slightly but should approximate 1800 bp. At this point, clones with abnormally sized inserts are easily identified and removed from further analysis.

The remainder of the PCR product is analyzed by RFLP. Three microliters of 42 mM MgSO<sub>4</sub>, 1  $\mu$ L *Bsu*RI (MBI Fermentas), and 1  $\mu$ L water are added to 10  $\mu$ L PCR product. *Bsu*RI, which recognizes the sequence GGCC, func-

tions over a wide range of buffer conditions, including the conditions found in the PCR. Hence, it is not necessary to clean the PCR product for the restriction digestion, but the final Mg<sup>2+</sup> concentration needs to be adjusted to 10 mM. The digestion is carried out at  $37^{\circ}\text{C}$  for 2 h, followed by electrophoresis of 5  $\mu$ L on a 3% NuSieve<sup>®</sup> agarose gel (BMA, Rockland, ME, USA). Sev-



**Figure 1. Major steps during the analysis of a 16S rRNA clone library starting with liquid cultures, including archiving, plasmid DNA preparation, PCR screening, and RFLP analysis.**

eral software programs, including FragmeNT (Amersham Pharmacia Biotech, Piscataway, NJ, USA) are available for sizing digestion products. The data are then compared, and the clones are distributed into like RFLP groups. A computer analysis of 16S rRNA sequences predicts an average of 4.80 restriction sites per gene, with a wide range of fragment sizes (6). *Bsu*RI cleaves in the multiple-cloning site of the vector so that very little of the vector is added to either the 5' or the 3' end of the cloned 16S rRNA gene (10 and 14 bases for pGEM-T Easy or 25 and 37 bases for pPCR II), thereby giving essentially identical RFLP patterns for identical genes cloned in opposite orientations. Clones belonging to the same RFLP group are compared in adjacent lanes of a second 3% NuSieve gel so that small differences in RFLP patterns can be easily detected. The use of the nearly complete 16S rRNA gene minimizes the chance that two clones from different phylogenetic groups will coincidentally have the same RFLP pattern. A second restriction enzyme, *Mbo*I (MBI Fermentas), which also functions in PCR buffer conditions, is used to verify the results of the first restriction. This enzyme also recognizes a 4-bp sequence, GATC, and is predicted to restrict 16S rRNA genes 3.97 times on average (6). A cocktail of 3  $\mu$ L 29 mM MgSO<sub>4</sub>, 1  $\mu$ L *Mbo*I restriction enzyme, and 1  $\mu$ L water is prepared for each member of an RFLP group. Again, there is no need to clean the templates, and the remaining 5  $\mu$ L PCR product can be digested in the original PCR tube using the same conditions as described earlier. A third 3% NuSieve gel is used to verify identical clones. Any clones that produce different RFLP patterns with the second enzyme are placed in a new RFLP group.

At this stage, all clones in the library with full-length inserts have been sorted into RFLP groups. Plasmids from representatives of each RFLP group can now be prepared using the pellets frozen from the initial overnight cultures. Plasmid yields from any of a number of commercially available kits should be sufficient to generate a high-quality template for the bidirectional sequencing of the entire insert. Standard 16S rRNA primers or commercial vector primers

can be used to obtain sequence, and data can be analyzed using standard methods.

Using RFLP analysis to sort the clones, Dunbar et al. (2) found that clones in the same RFLP group could range from 52.16% to 99.85% similarity, with an average similarity of 86.88%. It should be noted, however, that their analyses did not include a second gel in which clones with similar RFLP patterns were electrophoresed side by side. This additional step is very useful for detecting small differences in RFLP patterns that might be missed by relying on relatively imprecise data from image analysis programs. These programs are very useful for initial sorting, but the second gel often results in the identification of additional groups.

To verify the results of the RFLP analysis of a library of 53 clones, random representatives from three major RFLP groups were sequenced (Table 1). In Table 1, the roman numerals I, II, and III identify the major RFLP groups that were easily distinguishable through the examination of the initial RFLP analysis. After the first RFLP digestion, these groups contained 5, 7, and 7 members, respectively. After electrophoresing restriction digestion of these members side by side, and restriction analysis with a second enzyme, we discovered that some members had slightly different RFLP patterns. These were subsequently put into separate subgroups (Table 1; IB, IC, IIB, IIC, IIIB, and IIIC). Sequence analysis of the members of the main subgroups (Table 1; IA, IIA, and IIIA) confirmed that they were 98.7%–100% similar within the respective subgroup. Most of the clones, whose RFLP patterns were similar but not identical, were closely related (95.2%–100%) to the main subgroup. Subgroup IC, however, was only 79.3%–79.7% similar to subgroup IA, which is in the range reported by Dunbar et al. (2). These data indicate that a second RFLP gel with initially sorted clones electrophoresed side by side yields a more accurate RFLP sorting and is able to detect unrelated clones with coincidentally similar, but distinct, RFLP patterns. This method of screening is most useful for determining the dominant groups within a clone library.

Using this protocol, a library consisting of a few hundred clones can be

processed in less than a month with a minimal investment of materials and laboratory supplies. The initial isolated colonies are grown and aliquoted only once to minimize the chance of cross-contamination, while providing significant amounts of DNA for analysis. The DMSO plate can be used for the long-term storage of clones; alternatively, isolated plasmid DNA can be dried and retransformed as necessary. These general methods can be applied to a wide range of cloned PCR products and to 16S rRNA genes.

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## Specific Amplification of mRNA Splice Variants by RT-PCR

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Many eukaryotic proteins are encoded by multiple mRNA species generated by the alternative splicing of the same primary transcript. The quantitative measurement of these splice variants is essential to study their stability and kinetics of splicing. In some cases, they differ from each other in size by 30–40 bases. Under these circumstances, detection and/or quantitation of an individual splice variant in the presence of other(s) by northern blotting is not possible. The RNase protection assay, a very sensitive method for the detection of splice variants, usually involves the use of radiolabeled probes. Also, the radiolytic products of the probe sometimes mask protected bands, and, therefore, the results are not always conclusive. RT-PCR is a very rapid and sensitive technique that has been used for the detection of rare mRNA species. However, simultaneous amplification of several targets makes it difficult to quantitate and compare the level of a particular splice variant in different samples. We describe here a strategy based on RT-PCR by which any splice variant can be specifically amplified in the presence of others. This strategy is being demonstrated for a human lysosomal cysteine protease, cathepsin L and human vascular endothelial growth factor (VEGF) splice variants. Human cathepsin L is encoded by four mRNA species of which three (hCATL A, hCATL AI, and hCATL AII) are splice variants (1,4). hCATL A, the full-length form, includes all 278 bp of exon 1, whereas hCATL AI and AII lack 27 and 90 bases, respectively, of the 3' end of this exon (2). Similarly, human VEGF is encoded by four splice variants, namely VEGF<sub>206</sub>, VEGF<sub>165</sub>, VEGF<sub>145</sub>, and VEGF<sub>121</sub> (3). However, only VEGF<sub>165</sub> and VEGF<sub>121</sub> have the same donor sites and different acceptor sites, and, therefore, only these splice variants have been used to demonstrate this technique.

The rationale for designing PCR primers that could specifically amplify

each splice variant has been illustrated in Figure 1A. An antisense primer (SSC-21) was designed from exon 5 (a region common to all splice variants) and used for the amplification of each splice variant. The specific sense primers for each splice variant, encompassing the splice junction, consisted of 18 bases, of which the 6 bases at the 3' end corresponding to the splice acceptor site of exon 2 were the same. However, the sequence of the remaining 12 bases at the 5' end were different in primers for different splice variants and represented their respective splice donor sites from exon 1. Using this primer design, each sense primer could anneal along its entire length to only that template that contains its complementary sequence in a contiguous stretch. Thus, AG-5, AG-6, and AG-7 would specifically anneal to hCATL A, AI, and AII, respectively. A similar rationale was used for designing VEGF primers. However, in this case, a common sense primer (SSC-169) was used for both the splice variants, and the antisense primers spanning the splice junctions were different. SSC-170, an antisense primer specific for VEGF<sub>121</sub>, contained 12 bases reverse complementary to the 5' end of exon 8 at the 5' end and 6 bases at the 3' end that were reverse complementary to the 3' end of exon 5. The 6 bases at the 3' end of SSC-171, an antisense primer specific for VEGF<sub>165</sub>, were the same, and the remaining 12 bases at the 5' end were reverse complementary to the 5' end of exon 7.

To evaluate the specific amplification of splice variants by this strategy, we used SSC-21 in combination with AG-5, AG-6, or AG-7 for the amplification of hCATL A, AI, or AII and performed PCR with each set of primers and purified templates for the three splice variants separately. The PCR was carried out in a final volume of 50  $\mu$ L, containing 50 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton® X-100, 0.2 mM each dNTP, 2.5 U *Taq* DNA polymerase (Life Technologies, Rockville, MD, USA) and 40 pmol each primer. The cycling parameters were 20 cycles of denaturation, annealing, and extension at 94°C, 63°C, and 72°C, for 1 min each; 20 cycles of denaturation, annealing, and extension at 94°C, 55°C, and