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Combined Subtraction Hybridization and Polymerase Chain Reaction Amplification Procedure for Isolation of Strain-Specific *Rhizobium* DNA Sequences

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A novel subtraction hybridization procedure, incorporating a combination of four separation strategies, was developed to isolate unique DNA sequences from a strain of *Rhizobium leguminosarum* bv. trifolii. *Sau*3A-digested DNA from this strain, i.e., the probe strain, was ligated to a linker and hybridized in solution with an excess of pooled subtracter DNA from seven other strains of the same biovar which had been restricted, ligated to a different, biotinylated, subtracter-specific linker, and amplified by polymerase chain reaction to incorporate dUTP. Subtracter DNA and subtracter-probe hybrids were removed by phenol-chloroform extraction of a streptavidin-biotin-DNA complex. NENSORB chromatography of the sequences remaining in the aqueous layer captured biotinylated subtracter DNA which may have escaped removal by phenol-chloroform treatment. Any traces of contaminating subtracter DNA were removed by digestion with uracil DNA glycosylase. Finally, remaining sequences were amplified by polymerase chain reaction with a probe strain-specific primer, labelled with ^{32}P, and tested for specificity in dot blot hybridizations against total genomic target DNA from each strain in the subtracter pool. Two rounds of subtraction-amplification were sufficient to remove cross-hybridizing sequences and to give a probe which hybridized only with homologous target DNA. The method is applicable to the isolation of DNA and RNA sequences from both procaryotic and eucaryotic cells.

The fundamental principle in all subtraction hybridization protocols is the removal of nucleic acid sequences from one cell type or strain which are homologous with sequences from a different cell type. The nucleic acid to be subtracted is hybridized with an excess of subtracter sequences. Those sequences specific for a given strain, termed a probe strain, that have not hybridized with subtracter sequences are then isolated from the mixture, and a number of elaborate procedures for effecting this crucial separation have been described previously. In heterogeneous subtraction systems, the subtracter nucleic acid, which is immobilized on a solid support, is hybridized to probe strain DNA in the mobile phase, thus enriching that phase for probe strain-specific sequences which can be separated from the subtracter nucleic acid by centrifugation (3, 10). One problem with this method is leaching of the subtracter nucleic acid from the solid support into the mobile phase. In addition, a high percentage of the immobilized subtracter nucleic acid is rendered unavailable for hybridization because of steric hindrance (3).

Homogeneous subtractions utilizing single-phase hybridization and a variety of separation techniques have also been reported (2–5, 13). For example, hydroxylapatite chromatography can separate single- and double-stranded nucleic acids, assuming that cell-specific sequences remain single stranded (5). Disadvantages to this system are that single-stranded subtracter nucleic acid can contaminate the single-stranded probe fraction and that RNA containing extensive secondary structure can contaminate the double-stranded fraction (13). The system also requires large quantities of RNA to drive the subtraction event, which may be impractical unless an abundant source of tissue is available (7). The conversion of RNA to cDNA, followed by cloning, overcomes the last problem (6), and hydroxylapatite chromatography can be replaced by cupric-iminodiacetic acid chromatography to facilitate the separation of biotinylated subtracter nucleic acid from specifically enriched single-stranded sequences (13). Separation of biotinylated subtracter nucleic acid from cell-specific sequences can also be achieved by streptavidin-phenol-chloroform extraction, which is technically simple and has been used with success to isolate differentially expressed mRNA from mouse lymphoid cells and specifically expressed mRNA from *Xenopus* embryos (11). Batra et al. (2) used oligo(dA) affinity chromatography in conjunction with oligo(dT) tagged cDNA to isolate specific mRNA from an adenocarcinoma cell line, but this system requires an abundant source of RNA.

The techniques considered so far are frequently limited in their abilities to separate enriched cell-specific nucleic acid sequences from subtracter sequences by their reliance on single separation systems. Bjourson and Cooper (3) addressed this problem by using two-step affinity chromatography to separate biotinylated and mercurated subtracter DNA from specifically enriched bacterial DNA sequences by using streptavidin agarose and thiopropyl Sepharose. All previously reported subtraction techniques also require a subsequent cloning step, either for screening of genomic libraries to identify and facilitate isolation of particular nucleic acid fragments or to provide a renewable source of fragments for repeated use as probes.

In this paper, we report a novel subtraction system which utilizes a combination of four strategies for separating strain-specific *Rhizobium leguminosarum* bv. trifolii DNA se

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quences from subtracter DNA derived from a group of related strains. The method is applicable to the isolation of DNA and RNA sequences from a wide range of cell types.

MATERIALS AND METHODS

Origin of rhizobia. Eight *R. leguminosarum* bv. trifolii strains were obtained from the following sources: strain LPR5035, P. Hooykaas, Department of Biochemistry, University of Leiden, The Netherlands; strain ANU618, B. Rolfe, Research School of Biological Sciences, The Australian National University, Canberra, Australia; strain 14, S. Wright, United States Department of Agriculture, Beckley, W. Va. Strains P3, 1520, IDL, 1192, and 1312 were from our own culture collection.

Isolation of DNA. Rhizobia were individually grown in 50-ml conical flasks containing 25 ml of yeast extract mannitol broth (12) in a shaking incubator (150 rpm) at 25°C for 4 days. Five milliliters of each broth culture was centrifuged, and the pellets (50 μl) were transferred to 1.5-ml centrifuge tubes and then washed three times with 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and resuspended in 100 μl of a solution containing 25% sucrose, 1 mg of lysosyme ml^{-1} and 10 mM Tris-HCl (pH 8.0) for 15 min at 37°C. Cells were lysed by the addition of 200 μl of lysis solution (5 M guanidine isothiocyanate, 0.1 M EDTA [pH 7.0]). The lysate was mixed gently with 150 μl of 7.5 M ammonium acetate, and the mixture was extracted with 500 μl of chloroform-isoamyl alcohol (24:1, vol/vol) by mixing and centrifuging in a microcentrifuge for 10 min. The aqueous layer was transferred to a clean 1.5-ml centrifuge tube, and the DNA was precipitated by the addition of a 0.54 volume of 2-propanol. DNA was collected by spooling on a Pasteur pipette, washed twice in 100 μl of 70% ethanol, and resuspended in 20 μl of TE buffer. The concentration was estimated by gel electrophoresis of 5-μl aliquots (9). Approximately 1 μg of DNA from each strain was digested with restriction endonuclease *Sau*3A, and the restriction fragments were purified by using PREP-A-GENE matrix (Bio-Rad) as described by the manufacturer and resuspended in 20 μl of TE buffer. DNA from strain P3 was arbitrarily chosen as the source of strain-specific nucleic acid sequences, and this organism is subsequently referred to as the probe strain. The seven other strains were used as sources of subtracter DNA.

Synthesis of oligonucleotides and preparation of linkers. Oligonucleotides were synthesized by beta-cyanoethyl phosphoramidite chemistry using an Applied Biosystems model 380B DNA synthesizer (NICGENE, Belfast, Northern Ireland). Oligonucleotides had the following base sequences: TB7006, 5′ HO-AGCGGATAACAATTTCACACAGGA-OH 3′; TB7007, 5′ BIOTIN-CGCCAGGGUUUUCCCAGUCAC GAC-OH 3′; TB7008, 5′ P-GATCTCCTGTGTGAAATTGT-TATCCGCT-OH 3′; TB7009, 5′ P-GAUCGUCGUGACUG GGAAAACCCUGGCG-OH 3′. All oligonucleotides were resuspended in sterile TE buffer at a final concentration of 200 μM and stored in aliquots at −20°C. Five micrograms of each of the appropriate oligonucleotides was combined to produce the linkers L-P (TB7006 and TB7008) and L-S (TB7007 and TB7009). Mixtures were heated to 65°C and cooled slowly at room temperature to produce double-stranded linkers containing 5′-phosphorylated *Sau*3A-compatible overhangs at one end. Linker L-P was ligated to *Sau*3A-digested probe strain DNA (P3), and linker L-S was ligated to similarly digested subtracter DNA from the seven other strains. In all cases, *Sau*3A-digested DNA (200 ng) was mixed with 600 ng of the appropriate linker and the mixture was ligated with DNA ligase by using previously described methods (9). Excess linkers were removed with PREP-A-GENE matrix by using procedures described by the manufacturer, and linkered DNA was eluted in 20 μl of TE buffer at 50°C.

Preparation of probe strain DNA. One nanogram of strain P3 DNA modified by ligation to linker L-P was amplified by the polymerase chain reaction (PCR) by using 45 cycles with primer TB7006. Each cycle consisted of denaturation at 94°C for 1 min and 20 s, annealing at 55°C for 1 min, and DNA polymerization at 72°C for 2 min with an automated thermal cycler (model 480; Perkin-Elmer Cetus). Reactions were performed in sterile 0.5-ml tubes with 100-μl final reaction volumes containing the following in the concentrations indicated: Tris (pH 8.3), 10 mM; KCl, 50 mM; $MgCl_2$, 1.5 mM; gelatin, 0.01% (wt/vol); deoxynucleoside triphosphates, 200 μM; primer, 1 μM; 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Evaporation within the tubes was prevented by the addition of a 100-μl mineral oil overlay.

Preparation of subtracter DNA. Subtracter DNA from the seven strains, modified by ligation to linker L-S, was amplified by PCR as individual strain DNA by using the same amplification conditions described for probe strain DNA except that primer TB7007 was used instead of TB7006 and dUTP was substituted for dTTP to give a final dUTP concentration of 300 μM. The efficiency of amplification was examined by submitting 10-μl aliquots of each reaction mixture to gel electrophoresis with composite gels (3% NuSieve agarose–1% SeaKem agarose [wt/vol]; FMC BioProducts). Subtracter DNA PCR products were pooled, the mineral oil was removed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1), the aqueous phase was transferred to a Centricon 30 microconcentrator (Amicon Ltd., Stonehouse, United Kingdom), and the mixture was concentrated by spin dialysis to yield a final volume of 25 to 50 μl by using procedures described by the manufacturer. The volume was made up to 2 ml with 1 mM EDTA, and the spin dialysis step was repeated until the residual volume of the mixture was approximately 25 μl. This step was repeated with another 2 ml of 1 mM EDTA.

Biotinylation of subtracter DNA. While subtracter DNA sequences already contained a biotin moiety at their termini as a result of amplification with primer TB7007, additional biotinylation was performed to ensure that sufficient biotin molecules were present for subsequent binding to streptavidin and removal by phenol-chloroform extraction as described below. The pooled subtracter DNA was transferred to a 0.5-ml microcentrifuge tube (on ice), and 2 volumes of photobiotin acetate (Vector Laboratories, Peterborough, United Kingdom), was added. The mixture was placed at a distance of 10 cm from a 500-W mercury vapor lamp (Philips, Croydon, United Kingdom) and irradiated for 30 min. The reaction volume was made up to 100 μl with 0.1 M Tris-HCl, pH 9.0, and extracted twice with an equal volume of 2-butanol. Biotinylated subtracter DNA was precipitated at −70°C for 20 min after the addition of NaCl (200 μM final concentration) and 2 volumes of 99% (vol/vol) ethanol. The pellet was collected by centrifugation in a microcentrifuge (12,000 × *g* for 5 min), washed with 500 μl of 70% ethanol, dried under vacuum for 5 min, and redissolved in 10 to 15 μl of TE buffer.

Subtraction hybridization. The subtraction hybridization system is shown diagrammatically in Fig. 1. A 1- to 5-ng aliquot of PCR-amplified probe strain DNA and approximately 20 μg of subtracter DNA were mixed in a 0.5-ml

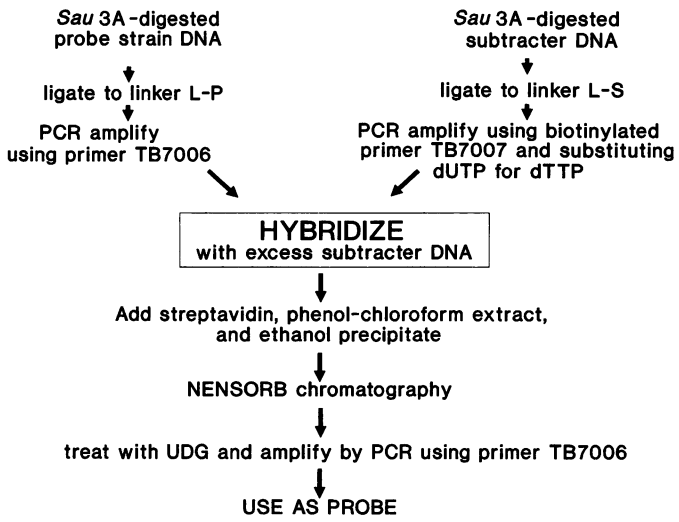

FIG. 1. Schematic representation for a single round of the combined subtraction hybridization and PCR amplification procedure.

microcentrifuge tube containing hybridization solution to give a total volume of 10 μl containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 500 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS) (final concentrations). The mixture was overlaid with 50 μl of mineral oil, and DNA was denatured at 99°C for 10 min, rapidly cooled on ice, and incubated at 64°C for 48 h to allow subtraction hybridization to take place.

Isolation of probe strain DNA sequences from the subtraction mixture. One hundred microliters of a solution containing 500 mM NaCl, 1 mM EDTA, and 50 mM HEPES was added, and the mixture was briefly centrifuged, excess mineral oil was removed, and 10 μg of streptavidin was added. The contents of the tube were mixed gently at room temperature for 5 min, and an additional 20 μg of streptavidin was added. The mixture was extracted with an equal volume of phenol-chloroform (50:50, vol/vol) and centrifuged in a microcentrifuge for 10 min at 12,000 rpm (12,700 × *g*). The aqueous phase was transferred to a fresh tube, SDS was added to 0.1%, and the mixture was extracted two more times with phenol-chloroform and once with chloroform. The DNA remaining in the aqueous phase was precipitated after the addition of 3 M sodium acetate (0.1 volume) and 2 volumes of 99% ethanol. The pellet, which was not visually discernible, was washed with 100 μl of 70% ethanol and redissolved in 20 μl of TE buffer, pH 8.0. The deproteinized DNA was desalted by using a NENSORB 20 purification cartridge (Du Pont Ltd., Stevenage, United Kingdom) as described by the manufacturers, and resuspended in 10 μl of TE buffer. Five microliters of this subtracted DNA, enriched for probe strain-specific sequences, was used for another subtraction cycle, and 1 μl was used to prepare logarithmic dilutions in 9-μl aliquots of TE buffer. Ten microliters of each dilution was mixed with PCR reagents (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin, 200 μM deoxynucleoside triphosphates) containing 15 U of uracil DNA glycosylase (UDG) (Cetus) and incubated at 37°C for 1 to 4 h to destroy any traces of dUTP-containing subtracter DNA. Five microliters of primer TB7006 (200 μM; probe strain specific) and 0.5 U of *Taq* polymerase were added to a final volume of 100 μl, and the reaction mixture was amplified for 45 cycles by using the temperature conditions described above for the preparation of probe strain DNA. PCR products were detected by electrophoresis of 10-μl aliquots of each PCR mixture in composite gels as described above.

The ability of the NENSORB cartridge to bind streptavidin-biotin-DNA complexes was also investigated, as follows. One microgram of biotinylated and unbiotinylated ^{32}P-labeled subtracter DNA was prepared by PCR amplification by using biotinylated primer TB7007 and a nonbiotinylated version of primer TB7007, respectively. Samples were mixed separately with 5 μg of streptavidin, and each was loaded onto NENSORB columns which were then washed and eluted as recommended by the manufacturer. Radioactivity in the eluted and irreversibly bound fractions was estimated by scintillation counting.

^{32}P-labelling of subtracted probe strain DNA. Five microliters of subtracted and PCR-amplified probe strain DNA was radiolabelled by using three cycles of amplification. Each cycle consisted of denaturation at 95°C (2 min), renaturation at 55°C (2 min), and polymerization at 72°C (10 min). Reactions were performed in 100-μl final volumes containing the following in the following concentrations: Tris (pH 8.3), 10 mM; KCl, 50 mM; $MgCl_2$, 1.5 mM; gelatin, 0.01% (wt/vol); dATP, dTTP and dGTP, 200 μM (each); 5 μl of [^{32}P]dCTP (400 Ci mM^{-1}); primer TB7006, 1 μM; and 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Unincorporated nucleotides were removed by using push column chromatography (Stratagene Ltd., Cambridge, United Kingdom).

Preparation of DNA dot blots. Total genomic target DNA (1 μg) from the appropriate rhizobia was denatured at 100°C for 10 min and rapidly cooled on ice, and 1 volume of 20× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) was added. Samples were manually spotted onto Hybond-N^+ membranes (Amersham Int.) in 5-μl aliquots. Membranes were placed on filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and transferred to another filter paper containing neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 1 mM EDTA) for 1 min. DNA was fixed to the membranes by placing them on filter paper soaked in 0.4 M NaOH (20 min), immersed briefly in 6× SSC, and air dried. Membranes were stored at 4°C until used.

Filter hybridizations. Filters were prehybridized for 5 h at 68°C in bags containing prehybridization solution (4× SSC, 10× Denhardt's solution, 100 μg of sonicated denatured salmon sperm DNA ml^{-1}, 10 mM Tris-HCl [pH 8.0], 0.1% SDS). Hybridization was performed at the same temperature with 2.5×10^6 cpm of subtracted DNA probe ml^{-1} for 18 h. Filters were washed in 1× SSC–0.5% SDS for 1 h (4×, 15 min) and in 0.1× SSC–0.5% SDS at 68°C for 1 h (4×, 15 min). Filters were subjected to autoradiography as previously described by Bjourson and Cooper (3).

RESULTS

Ligation of linkers, specificity of primers, and sensitivity of probe DNA amplification. Probe and subtracter DNAs were digested and ligated to linker sequences L-P and L-S, respectively, as described in Materials and Methods. The effect of the linkers on restriction fragments was examined by amplifying 1 μl of each ligation mixture; probe strain P3 DNA sequences were amplified with primer TB7006, while subtracter DNA was amplified with primer TB7007. Gel electrophoresis of aliquots of the completed PCR reaction mixtures produced smears of nucleic acid consistent with the

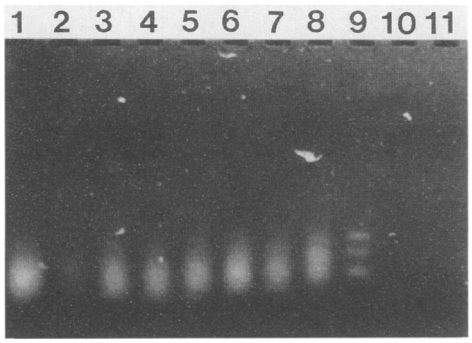

FIG. 2. Sensitivity of PCR amplification of probe strain DNA. Gel electrophoresis (3% NuSieve–1% SeaKem) of logarithmic dilutions of *R. leguminosarum* bv. trifolii strain P3 DNA ligated to linker L-P and PCR amplified with primer TB7006 (100 ng to 0.001 fg, lanes 1 to 11, with 10 μl of PCR reaction mixture per lane).

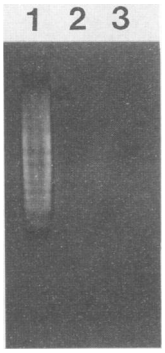

FIG. 3. Efficiency of streptavidin binding and phenol-chloroform extraction for the removal of biotinylated DNA. Nonbiotinylated subtracter DNA (lane 1) was generated by amplification with a nonbiotinylated version of primer TB7007, 5′-end biotinylated subtracter DNA (lane 2) was produced by PCR amplification with the biotinylated primer TB7007, or extensively biotinylated subtracter DNA (lane 3) was generated by PCR amplification with biotinylated primer TB7007 and additionally biotinylated with photobiotin acetate. In each case, 5 μg of DNA was mixed with 10 μg of streptavidin, extracted with phenol-chloroform (3×) and chloroform (1×), ethanol precipitated, resuspended in TE buffer, and electrophoresed in 3% NuSieve–1% SeaKem agarose gels.

size range exhibited by the nonlinkered, nonamplified, *Sau*3A-digested DNA, indicating that amplification was not biased with respect to sequence length over this fragment size range.

When amplification of *Sau*3A-digested DNA lacking linkers was attempted with either primer, no product was detected, as evidenced by the absence of a visible DNA smear when aliquots of the PCR reaction mixture were subjected to gel electrophoresis. This indicated that the products generated after amplification of linkered DNA were not due to nonspecific amplification but were totally dependent on the prior addition of linker sequences to the digested DNA (results not shown). When amplification of subtracter DNA containing the L-S linker was attempted with primer TB7006, no PCR products were generated. Similarly, when primer TB7007 was used to amplify probe strain DNA containing the L-P linker, no reaction products were detected. Logarithmic dilutions (100 ng to 00.1 fg) of probe strain DNA (P3) ligated to linker L-P were subjected to 45 cycles of amplification directed by primer TB7006 (Fig. 2). Amplification product was detectable by gel electrophoresis in the form of a smear when as little as 1 fg was used as the template. Below ca. 0.1 fg of template, PCR products were visible as a number of discrete bands, indicating that the template had been diluted to such an extent that only a few target molecules were present or that only a limited number of the template molecules present were susceptible to amplification by PCR. The smear in Fig. 2, lane 2, is of reduced intensity because of displacement of the sample from the well by traces of mineral oil. For subtraction hybridization purposes, it can be assumed that at least 1 fg of probe strain DNA sequences containing the linker L-P can be detected by PCR with primer TB7006.

Separation of probe strain-specific sequences from subtracter DNA. The efficiency with which phenol-chloroform extraction could remove biotinylated DNA-streptavidin complexes was examined (Fig. 3). Ten micrograms of 5′-end-labelled subtracter DNA was generated by using PCR amplification with the biotinylated primer TB7007. Five micrograms of this DNA was additionally labelled with photobiotin acetate. Streptavidin (5 μg) was added to each mixture, and the streptavidin-biotin-DNA complexes formed were removed by repeated phenol-chloroform extractions as described in Materials and Methods. DNA which remained in the aqueous phase in each case was ethanol precipitated and analyzed by gel electrophoresis. End labelling of DNA by PCR alone and streptavidin-phenol-chloroform extraction were sufficient to ensure removal of biotinylated sequences as seen by the absence of a smear (Fig. 3, lane 2).

NENSORB 20 cartridges were used primarily for desalting DNA solutions, but since the matrix irreversibly binds protein while permitting the recovery of DNA, their ability to remove biotin-DNA-streptavidin complexes was examined. The NENSORB matrix retained 96% of streptavidin-biotin-[^{32}P]DNA complexes and failed to retain 3.8%. The nonspecific binding of nonbiotinylated DNA by the matrix was low at 0.15%. NENSORB purification, in addition to the desalting of subtracted probe strain DNA, can therefore also serve to remove any subtracter DNA which may escape removal by streptavidin-phenol-chloroform extraction.

The effectiveness with which UDG was able to destroy subtracter DNA was examined. Subtracter DNA was prepared by PCR amplification so that all of the amplified sequences contained dUTP rather than dTTP (see Materials and Methods). When 10-μl aliquots of PCR products were treated with TE buffer (control) or 10 U of UDG (at 37°C for 4 h, 95°C for 10 min, and 55°C for 10 min) and the mixtures were examined by using gel electrophoresis, a DNA smear was visible in the control sample (Fig. 4, lane 1), while in the UDG-treated sample, DNA was not visually detectable (Fig. 4, lane 2).

Specificities of DNA sequences. The specificities of sequences remaining after subtraction hybridizations were assessed by dot blot hybridization against total genomic target DNA from the homologous probe strain, P3, and the seven other strains which composed the subtracter DNA. Results are shown in Fig. 5, which also demonstrates the cross-hybridizing properties of total genomic DNA from strain P3 with target DNA from the seven subtracter strains (Fig. 5A). After a single subtraction cycle followed by amplification of residual probe strain DNA with primer TB7006, sufficient nonspecific sequences were present to give a hybridization signal with target DNA from the sub-

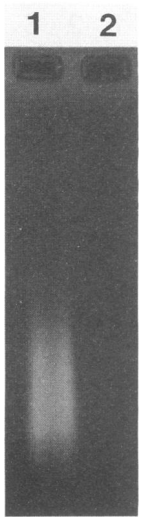

FIG. 4. Destruction of subtracter DNA by UDG. dUTP-containing subtracter DNA was prepared by PCR with primer TB7007 as described in Materials and Methods. A 10-μl aliquot was incubated at 37°C for 4 h with 10 μl of TE buffer (lane 1) or 10 μl (10 U) of UDG (lane 2) and then heated at 95°C for 10 min and incubated at 55°C for 10 min. The mixtures were analyzed by electrophoresis with composite 3% NuSieve agarose–1% SeaKem agarose gels.

tracter strains (Fig. 5B). A second subtraction-amplification cycle, however, generated a set of sequences which hybridized only with homologous P3 target DNA (Fig. 5C).

DISCUSSION

The subtraction hybridization method described here permits the reliable and reproducible isolation of DNA sequences which are unique to a given bacterial strain. The addition of the specific linker sequences to the probe strain and subtracter DNA prior to subtraction is a crucial component of the method design which confers a number of important advantages.

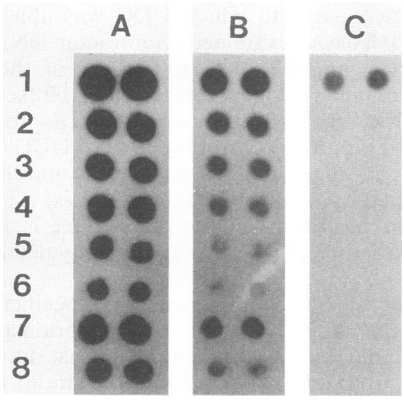

FIG. 5. Specificity of *R. leguminosarum* bv. trifolii strain P3 DNA sequences generated by subtraction hybridization. ^{32}P-labelled probe strain P3 DNA submitted to zero (A), one (B), or two (C) subtraction cycles was hybridized in duplicate to 1 μg of total genomic DNA from strains P3, IDL, 1192, 1520, 1312, ANU618, LPR5035, and 14 in lanes 1 to 8, respectively. For each subtraction cycle, the subtracter DNA was generated from the strains in lanes 2 through 8 as described in Materials and Methods.

For example, the subtracter DNA and the probe strain DNA can each be independently and specifically amplified by PCR. The results showed that modification of DNA by the addition of specific linkers permits the unbiased amplification of fragments in the 200- to 2,000-bp size range. This is in agreement with the results of other workers who reported previously that a highly complex mixture of cDNA tagged with lone linkers could be amplified in an unbiased fashion, provided that fragment lengths were less than 2.9 kb (8). Larger DNA fragments are also less efficiently amplified by *Taq* polymerase than are smaller fragments, resulting in a biased amplification favoring smaller sequences, although thermostable polymerases from different sources may vary in this respect. We used restriction enzyme *Sau*3A because it generated fragments in the 200- to 2,000-bp size range. When restriction enzyme *Bam*HI was used instead, the average fragment length was much larger, since this enzyme is a less frequent cutter than *Sau*3A. When *Bam*HI-digested DNA was ligated to specific linkers, amplification was biased in favor of the smaller fragment lengths (data not shown). We therefore recommend the use of restriction enzymes which generate DNA fragments with an average size of 2 kb or less. Another reason for using smaller DNA fragments is to avoid removal of potentially specific sequences which may be contained in a large fragment which also contains regions of homology with the subtracter DNA. For example, if two DNA fragments share 60% base sequence homology which is concentrated in one region, the hybrid formed between them will have a high melting temperature and be relatively stable. The nonhomologous region will be removed together with the homologous region during subtraction hybridization, particularly if low stringencies are employed. The use of smaller DNA restriction fragments reduces the risk of this occurring.

The ability to amplify probe strain DNA sequences after subtraction hybridization permits the use of less than 1 pg of modified probe strain DNA and a vast excess of subtracter DNA to drive the subtraction event. The use of such small amounts of probe strain DNA would not be possible with previously reported methods, since a large percentage of the starting material can be removed by subtraction hybridization, depending on the DNA base sequence homology between the subtracter DNA and probe strain DNA.

Despite the need for a large molar excess of subtracter DNA over probe strain DNA, the actual amount required for each subtraction was in the 10- to 20-μg range. Additionally, this subtracter DNA is in an easily renewable form, since an endless supply can be generated by PCR. This feature would assume special significance for organisms which are difficult to grow or whose DNA was difficult to extract. The method could be applicable to nonculturable organisms.

Another advantage of using small amounts of probe strain DNA against a large molar excess of subtracter DNA is that fewer subtractions are required, although this is also influenced by the degree of homology between subtracter and probe strain DNA. Welcher et al. (13) required five subtraction cycles when a 10-fold molar excess of subtracter DNA (*Neisseria meningitidis*) over probe DNA (*Neisseria gonorrhoeae*) was used to isolate *N. gonorrhoeae*-specific sequences, and screening of genomic libraries of these organisms was also required. In this study, only two subtraction cycles were necessary to obtain specific sequences for an *R. leguminosarum* bv. trifolii strain, even though the subtracter

DNA was composed of sequences from seven other strains within the same species.

The specificity of nucleic acid sequences generated during subtraction is dependent on the stringency at which the hybridization takes place. In this report, the subtraction hybridizations were carried out at 64°C, while dot blot hybridizations against the isolated specific sequences were performed at 4°C higher. The resulting sequences can therefore be regarded as highly specific for hybridization to homologous DNA at 68°C. The stringency at which the subtraction is performed is an important factor when considering the intended use of the final subtracted sequences; low-stringency subtraction will remove some probe strain sequences which have a relatively low base sequence homology with the subtracter DNA, whereas high-stringency subtraction will remove only perfectly matched sequences.

Efficient separation of probe strain-specific sequences from subtracter sequences and probe-subtracter hybrids is a prerequisite for a successful subtraction technique. This applies also to the enrichment of cDNA libraries for the isolation of specifically expressed mRNA from particular cell types. In this report, the probe strain-specific sequences were separated from biotinylated subtracter DNA and any probe strain DNA sequences hybridized to the subtracter sequences by a combination of four distinct strategies. (i) After subtraction hybridization, streptavidin was added to the hybridization mixture in order to bind to biotin moieties on the subtracter DNA. This protein-DNA complex partitions at the interface when extracted with phenol-chloroform, leaving probe strain-specific sequences in the aqueous layer. (ii) NENSORB chromatography was used primarily to desalt the probe strain DNA after each subtraction cycle, but this step also served as a useful supplementary binding step to capture biotinylated DNA, which may have escaped removal by phenol-chloroform extraction. (iii) Substitution of dTTP by dUTP in the biotinylated subtracter DNA permitted the destruction by UDG of any traces of subtracter DNA which had not been removed by the two previous separation procedures. (iv) Primer TB7006, which is specific for probe strain DNA sequences, was used for amplification of these sequences after each subtraction. This ensured that subtracter DNA which escaped removal by the other steps described above or which reentered the system as a contaminant was completely saturated by probe strain-specific sequences. The UDG digestion and specific amplification are useful features in that they are independent and are not affected by the degree of biotinylation, the UDG treatment acting primarily as a safeguard against contamination of probe strain sequences with subtracter DNA. Welcher et al. (13) found that the direct cloning of subtracted DNA was inefficient and was not reproducible because of variations in ligation and transformation efficiencies. This was not the case in our method, because specific amplification by PCR of the probe strain DNA enabled highly efficient direct cloning of the PCR products (data not shown). In addition, the ability to amplify the probe strain-specific sequences circumvents the requirement for screening of genomic or cDNA libraries.

The procedure described here is particularly suited to situations in which limited amounts of nucleic acid are available. Isolated DNA fragments, in addition to their use directly as probes, can be cloned as mentioned above and further characterized by sequencing or restriction mapping. Differentially expressed mRNA species in different cells or tissues could also be isolated by using this technique after conversion of the mRNA to cDNA. The addition of lone linkers after cDNA synthesis as previously described by other workers (1, 8) to permit cloning of small amounts of RNA could be used prior to subtraction.

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