A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn*5* insertion by the polymerase chain reaction

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ABSTRACT

We have developed a strategy to rapidly construct DNA hybridization probes for the isolation of genes disrupted by transposon Tn*5* insertions. A single oligonucleotide complementary to and extending outward from the ends of the inverted repeat of Tn*5* was used to prime DNA synthesis in the polymerase chain reaction. The amplified product consisted of DNA sequences adjacent to both ends of the transposon insertion. The general feasibility of the approach was tested by amplifying pBR322 sequences from a derivative of pBR322 containing a Tn*5* insertion. To amplify genomic DNA sequences flanking a Tn*5* insertion in the chromosome of a *Pseudomonas syringae* strain, circular substrates were generated by ligating *Eco*RI-digested genomic DNA. Tn*5* was contained intact within one such circular molecule, as the transposon does not contain sites for cleavage by *Eco*RI. The amplified product (~2.5 kb) was used as a DNA hybridization probe to isolate the homologous fragment from a cosmid library of wild-type *Pseudomonas syringae* genomic DNA. This approach may be applied to the efficient isolation of sequences flanking any Tn*5* insertion.

INTRODUCTION

The transposon Tn*5* has been used in genetic studies of a variety of gram-negative bacteria (1). It boasts the advantage of transposing efficiently in many bacterial species with little insertion specificity. The 5,820 base pair (bp) transposon is composed of 1.5 kilobase (kb) terminal inverted repeats of the insertion element IS*50* surrounding a central segment (1; M. P. Krebs and W. S. Reznikoff, personal communication). This internal portion of Tn*5* bears genes encoding resistance to several antibiotics, including kanamycin. Tn*5* insertions are flanked by 9 bp direct repeats of the sequence disrupted by the transposon.

We are interested in genes involved in the ability of *Pseudomonas syringae* strains to cause plant disease. The isolation of Tn*5* mutants unable to form disease lesions on host plant tissue has formed the basis of our genetic analysis. We sought to develop a procedure that would facilitate the molecular characterization of the mutations we have identified. This report describes a method to rapidly isolate target DNA sequences containing Tn*5* insertions (Figure 1). The approach is based on a variation of the polymerase chain reaction (PCR), termed inverse PCR or IPCR, in which the oligonucleotides priming DNA synthesis are oriented to extend outward from known sequences (7, 9, 10).

MATERIALS AND METHODS

Bacterial strains and plasmids

Pseudomonas syringae pathovar *syringae* strain B728a, a causal agent of bacterial brown spot disease of bean (*Phaseolus vulgaris*), was isolated from a Wisconsin field (S. S. Hirano, University of Wisconsin, Madison). Strain KW21 is a Tn*5* mutant of B728a that no longer forms disease lesions on bean (J. J. Rich, C. J. Kennedy, E. M. Hrabak and D. K. Willis, unpublished data). The plasmid pRZ705, a derivative of pBR322 (2) containing Tn*5* near the *Hin*dIII site at nucleotide position 29, was constructed by M. P. Krebs and obtained from V. Shultz and W. Reznikoff (University of Wisconsin, Madison).

Preparation of PCR templates

Plasmid DNA substrates for PCR amplification were purified by cesium gradient centrifugation. For chromosomal DNA PCR templates, mini-prep genomic DNA (3) was digested with *Eco*RI at 37°C for the minimum time necessary to achieve complete digestion (1 hour). The digestion mixture was ethanol-precipitated following heat inactivation of the enzyme. The digested DNA was then ligated in a 200 μl reaction containing 50 mM Tris-HCl pH 7.8, 5 mM $MgSO_4$, 10 mM dithiothreitol, 0.75 mM adenosine triphosphate, 50 μg/ml bovine serum albumin, and 200 U T4 DNA ligase (New England Biolabs) for 18 hours at 13°C. A DNA concentration of 5 μg/ml was sufficiently low to promote intramolecular rather than intermolecular ligation events. The ligation mixture was heat inactivated before ethanol-precipitating and resuspending in sterile water.

PCR conditions

The 20-base primer used for PCR amplifications was synthesized by the University of Wisconsin Biotechnology Center on an

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Applied Biosystems synthesizer. Plasmid DNA (10 ng) or genomic DNA prepared as described above (200–500 ng) was typically amplified in a 100 μl reaction containing 1× Promega *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 9, 0.01% gelatin (wt/vol), 0.1% Triton X-100, 1.5 mM $MgCl_2$), 200 μM final concentration of each dNTP, 2 μM final concentration of the single oligonucleotide primer, and 2.25 U of *Taq* polymerase (Promega). The reactions, minus the template, were routinely treated with UV irradiation for 5 minutes to destroy possible contaminating template. The 0.5 ml reaction tubes (Sarstedt) were placed on top of a Fotodyne transilluminator equipped with 305-nm bulbs (8) or within a Stratalinker 1800 UV crosslinker (254-nm bulbs) (Stratagene). These treatments were confirmed to prevent amplification of 50 ng of plasmid template DNA. The amplifications were performed using a Coy TempCycler machine, denaturing at 94°C for 1 minute, annealing at 55°C for 2 minutes (unless specified otherwise), and extending for 4 minutes at 72°C for 35 cycles.

Other methods

Amplified products of interest were excised from a 0.8% agarose gel in Tris-acetate/EDTA buffer (1× TAE) (6), electroeluted, and extracted once with chloroform before ethanol-precipitation. DNA concentrations were determined using a DNA fluorometer (Hoefer Scientific Instruments). The purified DNA was labeled with ^{32}P by priming DNA synthesis with random hexamers (Pharmacia) and the large (Klenow) fragment of DNA polymerase (International Biotechnologies, Inc.) (4). Labeled fragments were used to probe DNA transferred onto Zetabind nylon membrane (CUNO, Inc.) following the manufacturer's protocol. DNA sequencing reactions were performed with the T7 Sequencing Kit (Pharmacia LKB). 2 μg purified PCR product were sequenced using New England Biolabs primer #1204 (pBR322 *Eco*RI primer).

RESULTS

A 20-base oligonucleotide complementary to and extending outward from the inverted repeat of transposon Tn*5* was synthesized. The 20-mer (5′–GGTTCCGTTCAGGACGC-TAC–3′) is complementary to bases 37 to 18 of the left end of the top strand of Tn*5* and to bases 5784 to 5803 of the bottom strand of the right end of the transposon. The oligonucleotide is 60% G+C (*Tm*=61.4°C) and contains no stretches of homology longer than three bases within a single molecule or between two molecules. It was not possible to design an oligonucleotide closer to the ends of Tn*5* and still maintain these optimum characteristics.

To test whether the single primer could be used to amplify DNA flanking a Tn*5* insertion, a control experiment was performed using the plasmid pRZ705, a pBR322 derivative containing a Tn*5* insertion. Parallel PCR reactions were run using supercoiled pRZ705 or pBR322 as a template. As shown in Figure 2A, a ~4.4 kb product was amplified from pRZ705, while no product was amplified when pBR322 served as the template. The use of a linear substrate for amplification, formed by cleaving pRZ705 within Tn*5*, yielded slightly increased amounts of this DNA fragment (data not shown), as previously reported (9). The ~4.4 kb product amplified from pRZ705 was confirmed to hybridize to pBR322 sequences by Southern blot analysis (Figure 2B). Sequence analysis confirmed that the appropriate pBR322 and Tn*5* sequences were present in the

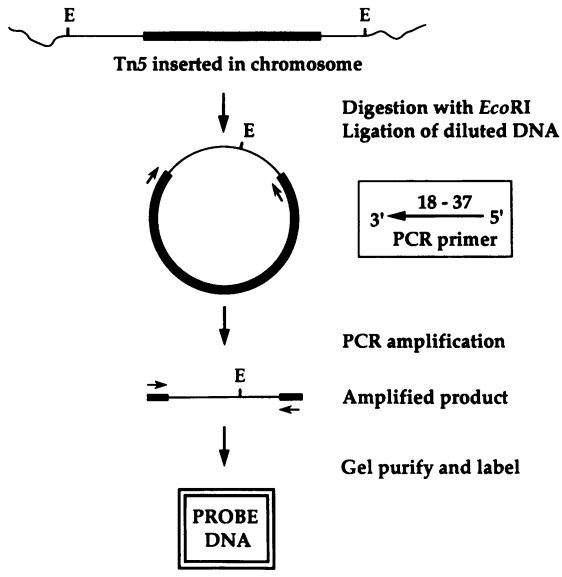

Figure 1. Strategy to amplify DNA sequences flanking a Tn*5* insertion. Chromosomal DNA is shown as a thin line, while Tn*5* sequences are depicted by a heavy line. Chromosomal *Eco*RI restriction sites (E) are indicated. The oligonucleotide primer for PCR, denoted by an arrow, is complementary to bases 18–37 of the top strand of Tn*5*.

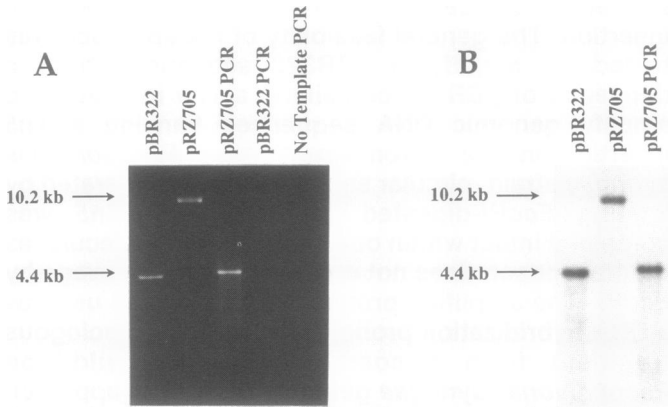

Figure 2. A) Ethidium bromide stained agarose gel showing PCR amplification of plasmid DNA flanking a Tn*5* insertion. Left two lanes show linearized plasmid DNA as labeled. The right three lanes show PCR products amplified from the substrates indicated. **B)** Autoradiograph of linearized plasmids and PCR product hybridized with the purified pRZ705 PCR fragment. Sizes of restriction fragments in kb are shown.

pRZ705 amplified fragment, and indicated that in pRZ705, Tn*5* was inserted at position 39 of pBR322. These results demonstrated that the single 20-mer could be used to amplify DNA sequences adjacent to both ends of a plasmid-borne Tn*5* insertion.

At $MgCl_2$ concentrations above 0.7 mM, fragments smaller than the desired product were amplified; the degree of amplification of the DNA fragment of interest did not vary appreciably from 0.7 to 1.9 mM $MgCl_2$ (data not shown). In each case described here, the desired fragment was sufficiently resolved from these artifact bands to allow uncontaminated excision from an agarose gel. Thus it was not necessary to

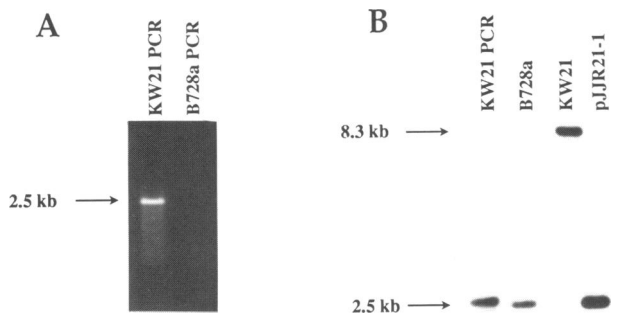

Figure 3. A) PCR amplification of sequences flanking the chromosomal Tn*5* insertion within strain KW21, shown by agarose gel electrophoresis. The control reaction used DNA from the parental strain B728a (lacking Tn*5*). **B)** Autoradiograph of *Eco*RI-digested genomic DNA (B728a and KW21) or plasmid DNA (pJJR21-1) using purified product amplified from strain KW21 as a hybridization probe. The probe DNA can be seen in the left-most lane.

optimize the $MgCl_2$ concentration for each PCR substrate, and was most convenient to use the *Taq* polymerase buffer supplied with the enzyme by the manufacturer (1.5 mM $MgCl_2$). In addition to the more typical, smaller molecular weight 'primer dimer', a product of ~0.5 kb (not shown) was observed in all amplifications including no template controls after destruction of possible contaminating template DNA by UV irradiation. Altering the concentration of $MgCl_2$ present in the PCR reaction or raising the annealing temperature from 55°C to 60°C did not significantly reduce the ~0.5 kb product. Lowering the primer concentration in the reaction, however, did reduce the amount of this product amplified. We concluded the unusually large ~0.5 kb product was likely to have derived from the primer.

We next sought to use the single primer to amplify sequences flanking the ends of a Tn*5* insertion in the chromosome of a strain of the gram-negative, plant pathogenic bacterium, *Pseudomonas syringae* pathovar *syringae*. The overall strategy is outlined in Figure 1. Firstly, genomic DNA of the Tn*5* mutant strain, KW21, was digested with *Eco*RI, an enzyme that does not cut within the transposon. The digested DNA was self-ligated at a DNA concentration low enough to favor intramolecular ligation. As a control, genomic DNA from the parental strain B728a (lacking Tn*5*) was prepared in a similar manner. These DNAs were used as substrates for PCR amplification using the single 20-mer to prime DNA synthesis. From previous Southern blot analysis using Tn*5* as a probe of *Eco*RI-digested KW21 chromosomal DNA, we knew the *Eco*RI target fragment containing Tn*5* was approximately 2.5 kb (data not shown). Thus we anticipated that a product of about 2.5 kb would be amplified only from the Tn*5*-containing template mixture. The agarose gel shown in Figure 3A shows that the expected results were obtained: a single band migrating at roughly 2.5 kb was amplified from the Tn*5* substrate (KW21) but not from the template lacking Tn*5* (B728a). Digestion of the ~2.5 kb amplified product with *Eco*RI yielded only two fragments (not shown), as predicted.

The ~2.5 kb fragment amplified from the Tn*5* mutant chromosomal DNA was purified, labeled with ^{32}P, and hybridized to a Southern blot of *Eco*RI-digested wild-type and Tn*5* mutant genomic DNA. As seen in Figure 3B, the probe hybridized to a ~2.5 kb fragment in the wild-type genome (B728a) and to a ~8.3 kb fragment in the Tn*5*-containing genome (KW21), corresponding to the target ~2.5 kb plus ~5.8 kb, the length of Tn*5*. This result demonstrated that the ~2.5 kb amplified product contained DNA sequences of the target *Eco*RI fragment.

We then made use of the PCR amplified probe to screen a cosmid library of wild-type genomic DNA. Out of 2208 colonies screened, 13 clones were confirmed to hybridize to the ~2.5 kb labeled probe. The ~2.5 kb *Eco*RI fragment appeared to be intact in 11 of these clones, while present in a truncated form in the remaining two. The result obtained with one of the former cosmids, designated pJJR21-1, is shown in Figure 3B. It is important to note that the amplified probe DNA was non-contiguous with respect to the original target *Eco*RI fragment; the rearranged nature of the DNA did not, however, diminish its utility as a hybridization probe.

DISCUSSION

This paper outlines a method to efficiently isolate DNA sequences adjacent to both ends of a chromosomal Tn*5* insertion. Using a single primer extending outward from the ends of the inverted repeat of the transposon, we amplified a probe consisting of DNA sequences of the entire target *Eco*RI fragment by PCR. Knowing the approximate size of this target fragment, the probe was generated with only a few short, simple manipulations. This approach should allow the use of any enzyme that does not digest Tn*5* and yields a target fragment short enough to be amplified. A variety of commonly used enzymes, e. g. *Eco*RI, *Eco*RV, *Kpn*I, etc., do not cut within Tn*5*. Cosmid clones identified by probing with PCR amplified sequences of the target *Eco*RV fragment of a different mutation complemented the defective phenotype of the corresponding Tn*5* mutant, further confirming the validity of this approach (J. J. Rich and D. K. Willis, unpublished data). While we have not established the maximum size of fragment that can be amplified by this method, other workers have amplified DNA up to 10 kb in length (5). It is encouraging for the general applicability of the method that the reactions described in this report gave the desired product on the first try and that this amplification was relatively insensitive to the different reaction conditions tested.

This strategy should be particularly convenient when a series of Tn*5* mutants are to be analyzed and assigned to linkage groups. It requires only one primer and many PCR reactions can be run at one time. The method is less costly both in time and in materials than conventional techniques for obtaining hybridization probes to identify clones containing undisrupted target DNA. These alternatives include cloning the target fragment by selecting the Tn*5* kanamycin resistance marker, for example, by constructing an individual genomic library for each Tn*5* mutant of interest. It is likely that a similar approach could be applied to the isolation of genes disrupted by transposable elements other than Tn*5*.

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