

## Characterization of Random Amplified Polymorphic DNA (RAPD) Products from *Xanthomonas campestris* and Some Comments on the Use of RAPD Products in Phylogenetic Analysis

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As part of our research to determine phylogenetic relationships of organisms within the phyto-bacterial species *Xanthomonas campestris*, we have examined the use of the random amplified polymorphic DNA (RAPD) technique. The objective of this aspect of our research was to determine if a valid cladistic character analysis could be carried out by direct comparison of RAPD products separated on ethidium bromide-stained agarose gels. RAPD products were amplified from 47 *Xanthomonas campestris* DNA templates using a single oligonucleotide primer. These RAPD products were compared and variation was characterized by Southern analysis of both RAPD products and genomic DNA of the 47 bacterial strains using two cloned RAPD products as probes. Analysis of the data set revealed that the RAPD products were not necessarily homologous or independent, crucial prerequisites for characters to be analyzed in a cladistic phylogenetic analysis. It has been commonly assumed that RAPD variation occurs due to insertion/deletion events or alterations in the primer binding site. Within our data set, we demonstrate absence phenotypes arising from the apparent absence of corresponding loci and also due to the preferred synthesis of alternative RAPD products from unrelated loci. These different types of variation are a reflection of different types of genotypic variation, and direct examination of RAPD products did not allow us to distinguish by which mechanism a particular absence phenotype arose. Although this may not be important for phenetic analyses, for analyses of homologous characters using a cladistic approach it is critical. We also detected unrelated, co-migrating RAPD products and multiple related RAPD products within reaction mixtures. These could both contribute to errors in estimates of similarity, important in any phylogenetic

analysis. All of these characteristics of RAPD products should be taken into consideration when RAPD products are used for phylogenetic comparisons. © 1994 Academic Press, Inc.

### INTRODUCTION

The random amplified polymorphic DNA technique (RAPD; Williams *et al.*, 1990) and the closely related arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) have come into broad use (see Hadrys *et al.*, 1992; Williams *et al.*, 1993, for reviews). These techniques have been successfully applied to the production of genetic linkage maps (Williams *et al.*, 1990; Klein-Lankhorst *et al.*, 1991) and the identification of genetic markers linked to certain phenotypes (Martin *et al.*, 1991; Michelmore *et al.*, 1991; Paran *et al.*, 1991). In addition, several workers have adapted the techniques for organism identification (Caetano-Anolles *et al.*, 1991; Goodwin and Annis, 1991) and resolution of taxonomic groups (Crowhurst *et al.*, 1991; Welsh *et al.*, 1992). However, since the nature of the variation uncovered using RAPD is not well characterized or understood (Hedrick, 1992), how to best use RAPD data for some applications, such as paternity determinations (Riedy *et al.*, 1992) and phylogenetic analyses (Welsh *et al.*, 1992; Kambhampati *et al.*, 1992; Tibayrenc *et al.*, 1993), remains a subject of debate.

We are interested in phylogenetic relationships within *Xanthomonas campestris*, a phytopathogenic bacterial species that is classified below the species level into more than 125 pathovars (*pathogenic variants* or *pv.*; Bradbury, 1984). We are studying these relationships in *X. campestris* *pv. poannua* (*pv. nov.*; Roberts *et al.*, in preparation), a bacterium that causes systemic wilt in annual bluegrass (*Poa annua* L.). Several strains of *X. campestris* *pv. poannua* are being developed in the United States for use as bioherbicides.

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It is therefore of interest to establish the phylogenetic placement of *X.c. pv. poannua* within *X. campestris*, identify the closest relatives of *X.c. pv. poannua*, and determine the potential for host range expansion of various *X.c. pv. poannua* strains.

This study was undertaken to determine how the RAPD technique might be used for these purposes. Most published phylogenetic analyses using RAPD data have used a phenetic approach; i.e., the data are used to establish pairwise similarity scores (genetic distances) between taxa (Kambhampati *et al.*, 1992; Grajal-Martin *et al.*, 1993; Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Tibayrenc *et al.*, 1993; Williams and St. Clair, 1993; Yang and Quiros, 1993). However, in two published studies (Welsh *et al.*, 1992; Landry *et al.*, 1993), a cladistic approach was taken, using RAPD bands as character data. Phylogenetic analysis in the cladistic sense requires the comparison of independent, homologous characters with genetically based variation (Hillis and Moritz, 1990; Wiley, 1981). We therefore sought to determine if RAPD products had these characteristics by obtaining data pertaining to the origin of the variation in RAPD banding patterns.

Our approach was to amplify DNA fragments from a collection of 47 *X. campestris* strains using a single oligonucleotide primer, RWII (5'-GGCCACCGTC-3'). Two of the resulting RAPD products were cloned and used as hybridization probes in Southern analyses of PCR products and genomic DNA. By restricting our analysis to a single primer and a relatively small number of strains, we have been able to characterize a set of RAPD products in some detail. By establishing the relationships of some of the RAPD products to each other, we obtained information about the nature of RAPD variation pertinent to the use of RAPD data in phylogenetic analysis.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Forty-seven *X. campestris* strains were used in this study (Table 1); these included eight isolates of *X.c. pv. poannua* from geographically distinct locations in seven states, two nonpathogenic *X. campestris* strains isolated from *P. annua* L., 23 strains of 12 other *X. campestris* pathovars that infect grasses, and 14 strains of *X. campestris* from nongramineae plant hosts. Bacteria were grown either on agar plates or in liquid culture in nutrient broth/yeast extract (NBY; Vidaver, 1967) medium at 28–30°C. Freezer stocks of cultures derived from single colony isolates were maintained at –70°C in NBY medium containing 15%(v/v) glycerol.

**DNA isolation from *X. campestris*.** Total genomic DNA was isolated from *X. campestris* using a modification of the protocol published by Meade *et al.* (1982). Cells from a 40-ml log-phase culture were washed

twice with 1 M NaCl and resuspended in 5 ml of TEE buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA). One milliliter of 10% (w/v) *N*-lauroyl sarcosine (Sigma) and 1 ml of Pronase (Boehringer Mannheim, 5 mg/ml, pre-digested at 37°C for 1 h) were added and incubated for 1 h at 37°C. One milliliter of 5 M NaCl and 1 ml of 10% (w/v) CTAB (cetyltrimethylammonium bromide, Sigma) dissolved in 0.7 M NaCl were added, mixed well, and incubated at 65°C for 10 min. Organic contaminants were removed from the cell lysate by one extraction with 5 ml of CHCl<sub>3</sub> and then several extractions with 5-ml aliquots of phenol-CHCl<sub>3</sub> (1:1; v/v). Nucleic acids were precipitated with 0.6 vol of isopropyl alcohol. The DNA was spooled out with a glass rod and dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The persistence of polysaccharide made some DNA samples highly viscous, and hence difficult to pipet accurately for quantitation. Some samples were therefore purified further by CsCl gradient centrifugation. This additional purification step had no obvious effect on the RAPD profile of a given strain.

In earlier stages of the work, DNA was quantitated spectrophotometrically ( $A_{260}$ ) and on ethidium bromide-stained agarose gels by comparison to standards of known mass. However, consistent quantitation was only achieved in later stages of the work, when DNA was quantitated by spectrofluorimetry at excitation and emission wavelengths of 365 and 460 nm, respectively, using the DNA-specific dye Hoechst 33258 (Hoefer Scientific) and a Model TKO-100 minifluorimeter (Hoefer Scientific) as specified by the manufacturer.

**Polymerase chain reaction.** PCR was carried out in a PE 9600 thermal cycler (Perkin-Elmer-Cetus) programmed as follows: 92°C, 4 min; 45 cycles of 92°C, 15 s; 37°C, 15 s; 72°C, 70 s; 72°C, 6 min 10 s. PCR reactions were performed in 25 µl containing 25 ng template DNA, 1.0 µM primer, 0.2 mM each dNTP, 0.8× AmpliTaq buffer (8 mM Tris, pH 8.3, 40 mM KCl, 1.2 mM MgCl<sub>2</sub>), and 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer-Cetus). These incubation times were recommended by Perkin-Elmer-Cetus technical support staff to mimic conditions used during preliminary reactions using a thermal cycler 480, which were those of Williams *et al.* (1990). A positive (enzyme) control was included in each set of reactions, containing 25 pg of λ DNA and a 1.0 µM concentration of each of two 14-nucleotide primers (primer 1, nucleotides 7142–7155 of (+) strand; primer 2, nucleotides 7606–7619 of (–) strand), which led to amplification of 500-bp fragment of λ DNA. A negative control was also included in each set of reactions using primer in the absence of DNA template.

Primers used in initial experiments were DDI, 5'-CGTACAAGAC-3'; JKI, 5'-TGACTCCGAG-3'; KSI, 5'-AGTGCCAGAG-3'; RWI, 5'-GTGCGAAGAC-3';

TABLE 1  
Description of *Xanthomonas* Strains Used in This Study

Strain	Identity	Host plant	Source (designation)
1	<i>X. campestris</i> pv. <i>poannua</i> ,* MI	<i>Poa annua</i> (annual bluegrass)	D. Roberts; Mycogen Corp. <sup>a</sup>
2	<i>X.c.</i> pv. <i>poannua</i> ,* PA	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp. <sup>b</sup>
3	<i>X.c.</i> pv. <i>poannua</i> ,* CA	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
4	<i>X.c.</i> pv. <i>poannua</i> ,* TN	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
5	<i>X.c.</i> pv. <i>poannua</i> ,* TX (Dallas)	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
6	<i>X.c.</i> pv. <i>poannua</i> ,* NY	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
7	<i>X.c.</i> pv. <i>poannua</i> ,* TX (Tyler)	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
8	<i>X.c.</i> pv. <i>poannua</i> ,* MO	<i>Poa annua</i> (annual bluegrass)	Mycogen Corp.
9	<i>X.c.</i> from <i>Poa annua</i> , AL		Mycogen Corp.
10	<i>X.c.</i> from <i>Poa annua</i> , LA		Mycogen Corp.
11	<i>X.c.</i> pv. <i>poae</i>	<i>Poa trivialis</i>	ATCC (33804); Mycogen Corp. <sup>c</sup>
12	<i>X.c.</i> pv. <i>graminis</i> ( <i>X. graminis</i> )	<i>Dactylis glomerata</i> (orchard grass)	ATCC (29091); Mycogen Corp.
13	<i>X.c.</i> pv. <i>graminis</i>	<i>Dactylis glomerata</i> (orchard grass)	ATCC (29087) <sup>d</sup>
14	<i>X.c.</i> pv. <i>graminis</i>	<i>Arrhenatherum elatius</i> (oat grass)	ATCC (29090)
15	<i>X.c.</i> pv. <i>graminis</i>	<i>Festuca pratensis</i> (fescue)	D. Roberts (Xg723) <sup>e</sup>
16	<i>X.c.</i> pv. <i>graminis</i>	<i>Lolium multiflorum</i> (It. rye grass)	D. Roberts (Xg726)
17	<i>X.c.</i> pv. <i>graminis</i>	<i>Phleum pratense</i> (timothy)	D. Roberts (Xg729)
18	<i>X.c.</i> pv. <i>digitarii</i> ,* WI	<i>Digitaria</i> sp. (crabgrass)	Mycogen Corp.
19	<i>X.c.</i> pv. <i>agrostis</i> ,* IL (St. Charles)	<i>Agrostis</i> sp. (cr. bentgrass)	D. Roberts
20	<i>X.c.</i> pv. <i>agrostis</i> ,* IL (Chicago)	<i>Agrostis</i> sp. (cr. bentgrass)	D. Roberts
21	<i>X.c.</i> pv. <i>agrostis</i> ,* OH (Dublin)	<i>Agrostis</i> sp. (cr. bentgrass)	D. Roberts
22	<i>X.c.</i> pv. <i>translucens</i>	Unknown	ATCC (9000)
23	<i>X.c.</i> pv. <i>translucens</i>	<i>Hordeum vulgare</i> (barley)	ATCC (10731)
24	<i>X.c.</i> pv. <i>translucens</i>	<i>Hordeum vulgare</i> (barley)	ATCC (10770)
25	<i>X.c.</i> pv. <i>translucens</i>	<i>Triticum</i> sp. (wheat)	ATCC (10771)
26	<i>X.c.</i> pv. <i>translucens</i>	<i>Secale</i> sp. (rye)	ATCC (10772)
27	<i>X.c.</i> pv. <i>arrhenatheri</i>	<i>Arrhenatherum elatius</i> (oat grass)	ATCC (33803); Mycogen Corp.
28	<i>X.c.</i> pv. <i>holcicola</i>	<i>Holcus</i> sp. (velvet grass)	ATCC (13461); Mycogen Corp.
29	<i>X.c.</i> pv. <i>phlei</i>	<i>Phleum pratense</i> (timothy)	ATCC (33805); Mycogen Corp.
30	<i>X.c.</i> pv. <i>secalis</i>	<i>Secale</i> sp. (rye)	ATCC (49078)
31	<i>X.c.</i> pv. <i>oryzae</i>	<i>Oryza sativa</i> (rice)	ATCC 43837
32	<i>X.c.</i> pv. <i>oryzicola</i>	<i>Oryza sativa</i> (rice)	ATCC (49072)
33	<i>X.c.</i> pv. <i>vasculorum</i>	<i>Saccharum officinarum</i> (sugar cane)	ATCC (35938)
34	<i>X.c.</i> pv. <i>dieffenbachiae</i>	<i>Dieffenbachia</i> sp.	J. Hartung (X11) <sup>f</sup>
35	<i>X.c.</i> pv. <i>campestris</i>	<i>Brassica oleraceae</i> var. <i>gemmifera</i>	ATCC (33913)
36	<i>X.c.</i> pv. <i>campestris</i>	<i>Brassica</i> sp.	J. Hartung (X6)
37	<i>X.c.</i> pv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i> (bean)	ATCC (9563)
38	<i>X.c.</i> pv. <i>phaseoli</i>	<i>Phaseolus vulgaris</i> (bean)	J. Hartung (X35)
39	<i>X.c.</i> pv. <i>alfalfae</i>	<i>Medicago sativa</i> (alfalfa)	J. Hartung (X60)
40	<i>X.c.</i> pv. <i>vesicatoria</i>	<i>Lycopersicon lycopersicum</i> (tomato)	ATCC (35937); Mycogen Corp.
41	<i>X.c.</i> pv. <i>begoniae</i>	<i>Begonia</i> sp.	J. Hartung (X3)
42	<i>X.c.</i> from <i>Bilvae</i>		J. Hartung (X32)
43	<i>X.c.</i> from <i>Feronia</i>		J. Hartung (X33)
44	<i>X.c.</i> pv. <i>citri</i> "A"	<i>Citrus</i> sp.	J. Hartung (XC62)
45	<i>X.c.</i> pv. <i>citri</i> "B"	<i>Citrus</i> sp.	J. Hartung (XC84)
46	<i>X.c.</i> pv. <i>fici</i>	<i>Fiscus</i> sp.	J. Hartung (X151)
47	<i>X.c.</i> pv. <i>malvacearum</i>	<i>Gossypium</i> sp.	J. Hartung (X203)

\* Pathovar names are tentative.

<sup>a</sup> Bacterial culture obtained either directly from D. Roberts (Plant Path., Mich. St.) or from D. Roberts via Mycogen.

<sup>b</sup> Bacterial culture obtained from Mycogen Corp., San Diego, CA.

<sup>c</sup> Bacterial culture obtained either directly from ATCC or from ATCC via Mycogen.

<sup>d</sup> Bacterial culture obtained from ATCC.

<sup>e</sup> Bacterial culture obtained from D. Roberts.

<sup>f</sup> Bacterial DNA obtained from J. Hartung (USDA, Beltsville, MD).

and RWII, 5'-GGCCACCGTC-3'. These were the kind gift of Rick Ward in the Crop and Soil Science Department at Michigan State University. Additional primers were later synthesized for us at the MSU Macromolecular Structure and Sequence Facility.

If PCR products were to be cloned, the thermal cycler program was appended, immediately following the 6 min 10 s elongation at 72°C, to include a 5-min inactivation of the *Taq* polymerase at 99°C. DNA was then reannealed by cooling to 25°C over a 30-min period. The ends of the PCR products were made blunt by raising the MgCl<sub>2</sub> concentration to 5 mM, adding 1 unit of the Klenow fragment of DNA polymerase (Boehringer Mannheim), and incubating the resulting mixture for 30 min at RT. The Klenow fragment was then heat inactivated at 65°C for 10 min and the reaction placed on ice.

**Cloning of PCR products.** PCR products for cloning were gel-purified by electroelution (Sambrook *et al.*, 1989), precipitated with EtOH, resuspended in TE, and quantitated. Products were then cloned by blunt-end ligation to *Sma*I-digested phagemid pBSII KS<sup>-</sup> (Stratagene) with 1 unit of T4 DNA ligase (Boehringer Mannheim) using 200 ng of gel-purified PCR product and 100 ng of *Sma*I-digested vector in a 20- $\mu$ l reaction. The ligation reaction products were used to transform *Escherichia coli* (DH5 $\alpha$ ; Gibco BRL), and putative positive clones were screened for inserts of the appropriate molecular weight. Cloning of the 1.2 and 1.8-kb RAPD products from the MI strain of *X.c. pv. poannua* yielded the recombinant plasmids pJRL100 and pJRL200, respectively.

**Digoxygenin labeling of DNA.** DNA inserts from pJRL100 and pJRL200 were gel-purified by electroelution after double digestion with *Hind*III and *Not*I. Purified DNA inserts (100 ng) were labeled with digoxigenin-dUTP by the random primer method (Feinberg and Vogelstein, 1984) using a commercially available kit (Boehringer Mannheim). Total yield from the labeling reaction (80  $\mu$ l volume) was calculated to be 100–150 ng of labeled DNA using the yield chart in Boehringer Mannheim Technical Bulletin 9009962/5M (January 1991).

**Southern analysis.** *Eco*RI-digested genomic DNA fragments were separated by agarose gel electrophoresis and transferred to a GeneScreen hybridization membrane (DuPont, Inc.) using the procedure described in Sambrook *et al.* (1989). Blots were treated with hybridization buffer [5  $\times$  SSC, 1% (w/v) casein (Sigma C5890), 0.1% (w/v) *N*-lauroyl sarcosine, and 0.2% (w/v) SDS] for 2–4 h, and then hybridization was carried out for 12–16 h in fresh buffer containing approximately 5 ng labeled DNA/ml. Blots were washed twice at RT for 5 min with 2  $\times$  SSC/0.1% SDS and then twice for 15 min at various stringencies (see figure leg-

ends). Hybridized DNA was detected by exposing blots to Kodak XAR-5 film after incubation with the anti-digoxygenin alkaline phosphatase conjugate (50 mU/ml) and LumiPhos substrate of the Genius System (Boehringer Mannheim) using the protocol outlined in the Boehringer Mannheim Technical Bulletin for LumiPhos 530 (900264R3/10M; January 1991), substituting casein for blocking reagent. When blots were to be reprobated, probes were stripped by boiling for 30 min in 0.01  $\times$  SSPE/1.0% SDS.

## RESULTS

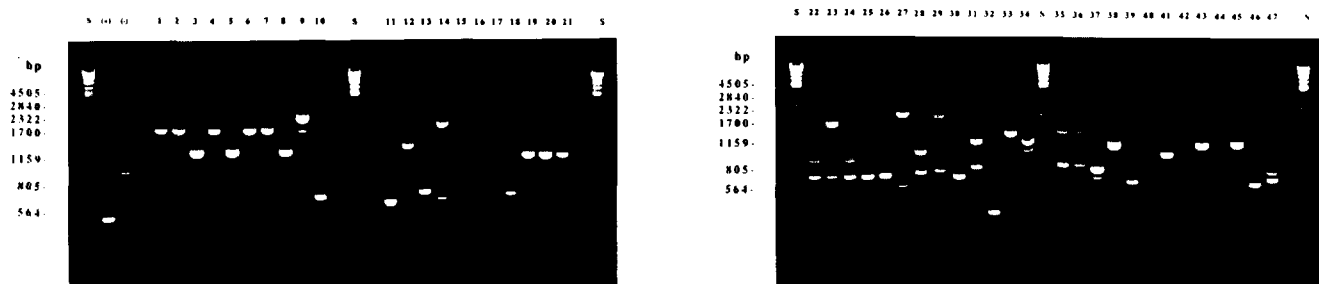
### *RAPD Products and RAPD Product Profiles*

In preliminary experiments, we tested five arbitrarily chosen 10-bp oligonucleotides for RAPD amplification of DNA from *X.c. pv. poannua*. Two of the five primers (DDI and RWII) yielded measurable products, and RWII was chosen for use in the experiments described below.

Amplification of DNA fragments was observed in all 47 *X. campestris* strains when RWII was used as primer (Fig. 1). In most strains, one or two major products dominated the profile, with several minor products also appearing (e.g., *X.c. pv. poannua*, strains 1–8). However, in some cases, a single product stood out (e.g., *X.c. pv. agrostis*, strains 19–21; *X. campestris* from *Festonia* sp., strain 43; and *X.c. pv. citri* "B," strain 45) or many products were amplified with none predominating (e.g., *X.c. pv. graminis*, strains 15 and 16; *X.c. pv. campestris*, strain 36; *X.c. pv. vesicatoria*, strain 40; and *X.c. pv. citri* "A," strain 44). Differences as well as similarities were observed in profiles from strains of the same pathovar, in particular *X.c. pv. graminis* (strains 12–17), *X.c. pv. translucens* (strains 22–26), and *X.c. pv. poannua* (strains 1–8).

Interestingly, *X.c. pv. graminis* (strain 14) and *X.c. pv. arrhenatheri* (strain 27), both isolated from *Arrhenatherum elatius*, had almost identical RWII-primed RAPD products (Fig. 1). Likewise, *X.c. pv. graminis* (strain 17) and *X.c. pv. phlei* (strain 29), both isolated from *Phleum pratense*, had almost identical RWII-primed RAPD products, as did *X.c. pv. translucens* (strain 26) and *X.c. pv. secalis* (strain 30), both isolated from *Secale* sp.

Two distinct RAPD product patterns were observed in the eight *X.c. pv. poannua* strains (Fig. 1); in five strains a 1.8-kb product predominated, while in the other three strains, the 1.8-kb product was absent and a 1.2-kb DNA predominated. To study the relationship of these two RAPD products to each other and to the other RAPD products amplified from the other *X. campestris* strains, the 1.2 and 1.8-kb RAPD products were isolated from *X.c. pv. poannua* (strain 1) and cloned to yield the plasmids pJRL100 and pJRL200, respectively.

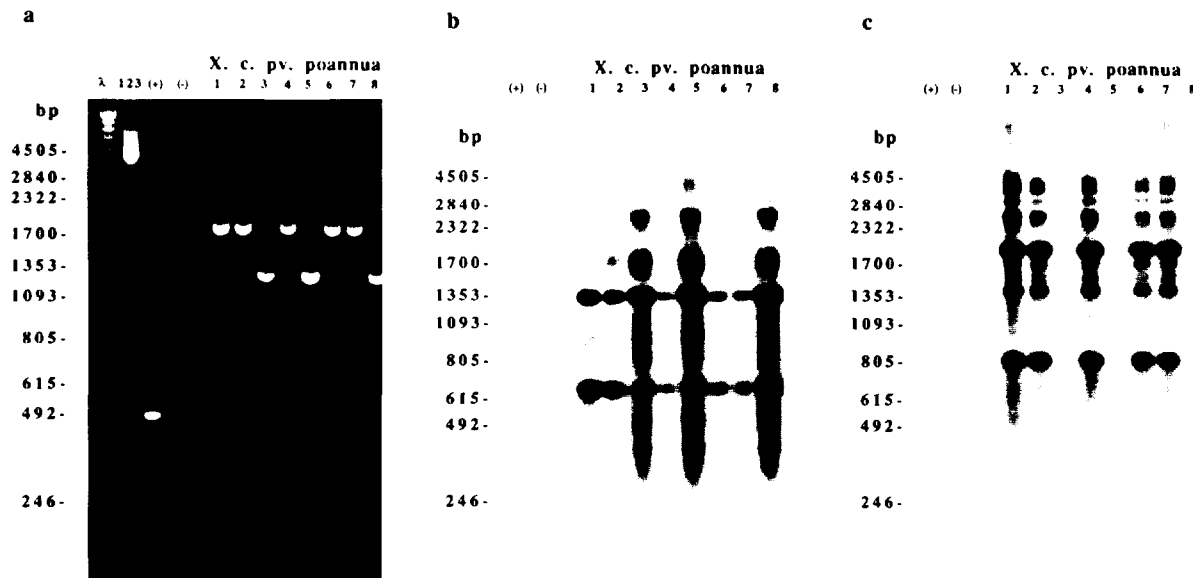


**FIG. 1.** Random amplified polymorphic DNA (RAPD) products from 47 *Xanthomonas campestris* strains. Agarose gel (1.2%) of DNA products obtained upon RWII priming of the PCR with 47 bacterial DNA templates. Sample load was 5  $\mu$ l/25  $\mu$ l total reaction. Lanes (numbers also refer to Table 1): 1–8, *X.c. pv. poannua*; 9 and 10, *X. campestris* from *Poa annua* L.; 11, *X. campestris pv. poae*; 12–17, *X. campestris pv. graminis*; 18, *X. campestris pv. digitarii*; 19–21, *X. campestris pv. agrostis*; 22–26, *X. campestris pv. translucens*; 27, *X. campestris pv. arrhenatheri*; 28, *X. campestris pv. holcicola*; 29, *X. campestris pv. phlei*; 30, *X. campestris pv. secalis*; 31, *X. campestris pv. oryzae*; 32, *X. campestris pv. oryzicola*; 33, *X. campestris pv. vasculorum*; 34, *X. campestris pv. dieffenbachiae*; 35 and 36, *X. campestris pv. campestris*; 37 and 38, *X. campestris pv. phaseoli*; 39, *X. campestris pv. alfalfae*; 40, *X. campestris pv. vesicatoria*; 41, *X. campestris pv. begoniae*; 42, *X. campestris* from *Bilvae*; 43, *X. campestris* from *Feronia*; 44 and 45, *X. campestris pv. citri*; 46, *X. campestris pv. fici*; 47, *X. campestris pv. malvacearum*; S, MW standards, a mixture of *Hind*III-digested  $\lambda$  DNA and *Pst*I-digested  $\lambda$  DNA; (+), positive control,  $\lambda$  DNA amplified with  $\lambda$  DNA primers designed to give a 500-bp product; (–), negative control using RWII primers with buffer only as template.

*Characterization of pJRL100 and pJRL200*

Southern blots of the RAPD products from the *Xanthomonas* strains demonstrated the correct identity and uniqueness of the cloned 1.2-kb (pJRL100) and 1.8-kb (pJRL200) RAPD products from *X.c. pv. poannua* (Fig. 2). The pJRL100 insert hybridized to the RAPD products of all eight *X.c. pv. poannua* strains (Fig. 2b). This was somewhat surprising, since the presence of

a 1.2-kb band was not obvious from examination of EtBr-stained agarose gels (Fig. 1 and Fig. 2a). However, closer examination of these gels reveals a band of the correct MW in *X.c. pv. poannua* strains 1, 2, 4, 6, and 7), and the Southern hybridization signal tended to approximate the intensity of the EtBr-stained band (Fig. 2b). The pJRL200 insert hybridized to the RAPD products only from *X.c. pv. poannua* strains 1, 2, 4, 6,



**FIG. 2.** Southern analysis of RAPD products from eight *X.c. pv. poannua* strains. RAPD products were blotted and probed with either the pJRL100 or pJRL200 insert. (a) Agarose gel of RAPD products. (b) Probe with the pJRL100 insert. Note hybridization to at least two DNA fragments in all eight *X.c. pv. poannua* strains. (c) Probe with the pJRL200 insert. Note hybridization to at least three different DNA fragments, and in only five of the eight *X.c. pv. poannua* strains. Neither probe hybridized to any fragments in the positive or negative control lanes. Samples: (a) 5  $\mu$ l/25  $\mu$ l total reaction in each lane; (b) and (c) 0.5  $\mu$ l/25  $\mu$ l total reaction. Standards:  $\lambda$ , a mixture of *Hind*III-digested  $\lambda$  DNA and *Pst*I-digested  $\lambda$  DNA; 123, 123-bp ladder (Gibco BRL); (+), positive control,  $\lambda$  DNA amplified with  $\lambda$  DNA primers designed to give a 500 bp product; (–), negative control using RWII primers with buffer only as template. Hybridization, 65°C; washes, 0.2 $\times$  SSC/0.1% SDS, 65°C; film exposures, 1 min.

and 7 (Fig. 2c) and not at all to the RAPD products of *X.c. pv. poannua* strains 3, 5, and 8. This pattern mimics exactly the pattern observed on the EtBr-stained agarose gel (Fig. 2a).

The pJRL100 insert hybridized only to the RAPD products of the *X.c. pv. poannua* strains. It did not hybridize at all to the RAPD products from the other 39 *X. campestris* strains (negative results not shown). On the other hand, in addition to hybridizing to the RAPD products of five of the eight *X.c. pv. poannua* strains, the pJRL200 insert hybridized to the RAPD products of *X. campestris* from *P. annua*, strain 9; *X.c. pv. graminis*, strain 13; *X.c. pv. dieffenbachiae*, strain 34; *X.c. pv. campestris*, strain 36; and *X.c. pv. phaseoli*, strain 37 (not shown).

The pJRL100 and pJRL200 inserts were also characterized by hybridization to more than one RAPD product. pJRL100 hybridized not only to the RAPD product at 1.2 kb but also to another at approximately 0.7 kb (Fig. 2b), while pJRL200 hybridized to at least two products (approximately 1.3 and 0.8 kb) in addition to the 1.8-kb product (Fig. 2c). Significantly, these hybridizing sets of RAPD products did not intersect; individual RAPD products hybridized to either the pJRL100 insert or the pJRL200 insert, but no RAPD product hybridized to both probes (cf. Figs. 2b and 2c). Thus, we concluded that the pJRL100 and pJRL200 inserts were unique DNA fragments, representing DNA amplification from two separate genome locations.

Preliminary Southern analysis of chromosomal and purified plasmid DNA from *X.c. pv. poannua* suggested that both the 1.2-kb pJRL100 insert and the 1.8-kb pJRL200 insert are on the bacterial chromosome and not plasmid-borne (not shown).

#### Genomic Southern Analysis

To attempt to determine the basis of the observed variation in the RAPD banding patterns, Southern blots of *EcoRI*-digested total genomic DNA from the 47 bacterial strains were probed with the purified inserts from pJRL100 and pJRL200 (Fig. 3). This analysis allowed us to distinguish between band absence arising from complete absence of a given RAPD locus and absence due to primer binding site differences, insertion/deletion events, or other causes (such as competition; see below). The pJRL100 insert hybridized to an approximately 23-kb *EcoRI* fragment in the DNA of all eight *X.c. pv. poannua* strains (7 of 8 shown in Fig. 3b).

This result showed that the reduction in band intensity (and sometimes complete absence) of the 1.2-kb fragment in the RAPD products from *X.c. pv. poannua* strains 1, 2, 4, 6, and 7 was not due to the absence of a potentially amplifiable fragment from the genomes of these strains. On the other hand, the pJRL100 insert did not hybridize to the genomic DNA of any of the other 39 bacterial strains (negative results not shown), suggesting that a homologous DNA fragment corresponding to the 1.2-kb RAPD product was either absent from the genomic DNAs of these strains or has diverged to a degree such that it no longer hybridizes to the pJRL100 insert.

Genomic Southern analysis with the pJRL200 probe (Fig. 3c) divided the eight *X.c. pv. poannua* strains into three categories: those with a hybridizing *EcoRI* fragment of 4.6 kb (strains 1, 4, 6, and 7); those with a fragment of 9.4 kb (strain 2); and those lacking a hybridizing fragment (strains 3, 5, and 8). Thus, the absence of a 1.8-kb RAPD product in *X.c. pv. poannua* strains 3, 5, and 8 was most likely due to the absence of a potentially amplifiable DNA fragment in these genomes.

The pJRL200 insert also hybridized, depending upon stringency, to *EcoRI* fragments of *X. campestris* from *P. annua*, strain 9 (Fig. 3c); *X.c. pv. graminis*, strains 12 and 13; *X.c. pv. holcicola*, strain 28; *X.c. pv. oryzicola*, strain 32; *X.c. pv. dieffenbachiae*, strain 34; *X.c. pv. campestris*, strain 36; *X.c. pv. phaseoli*, strains 37 and 38; *X.c. pv. alfalfae*, strain 39; *X.c. pv. begoniae*, strain 41; *X.c. pv. citri* "A," strain 44; and *X.c. pv. malvacearum*, strain 47 (not shown). Only a subset of these strains yielded RAPD products that hybridized to the pJRL200 probe (see above).

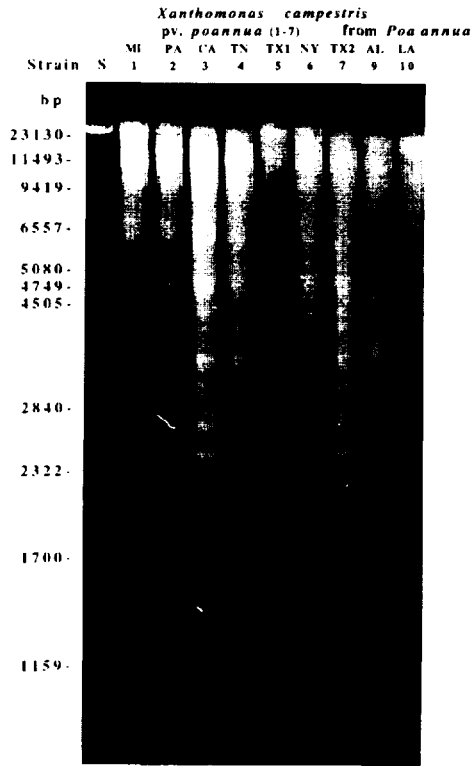
To verify the identity of certain strains as *X. campestris*, some Southern blots were probed with a 9.6-kb *HindIII*-*Bam*HI fragment from pIG102 (Fig. 3d), which encodes part of a gene cluster involved in xanthomonadin biosynthesis (Poplawsky *et al.*, 1993). All eight *X.c. pv. poannua* strains contained *EcoRI* fragments that hybridized to this fragment, as did the two nonpathogenic *X. campestris* strains from *P. annua*.

#### Genomic DNA Mixing Experiment

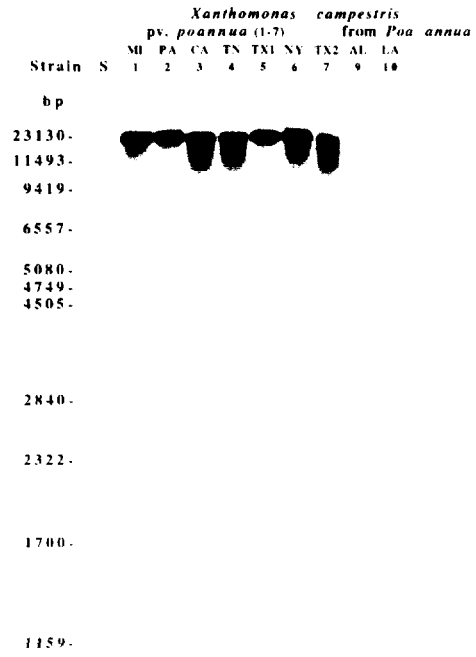
The above results suggested that a DNA fragment corresponding to the 1.2-kb RAPD product was present in the genomes of *X.c. pv. poannua* strains 1, 2, 4, 6, and 7 (Fig. 3b), and that it could be amplified (Fig. 2b). Yet for some reason, amplification of this fragment in

**FIG. 3.** Genomic Southern analysis of nine *Xanthomonas campestris* strains isolated from *Poa annua*. Seven of the nine strains shown (1–7) are pathogenic on *Poa annua* (*X.c. pv. poannua*). The two other strains (9 and 10) are nonpathogenic *X. campestris* from *Poa annua*. (a) Agarose gel (0.7%) of *EcoRI*-digested total genomic DNA (~ 2.5 µg). (b) Southern blot probed with the pJRL100 insert shows specificity of this DNA fragment for *X.c. pv. poannua*. Hybridization, 68°C; washes, 0.1 × SSC/0.5% SDS, 68°C; film exposure, 3 min. (c) Blot in (b) stripped and reprobed with the pJRL200 insert. Hybridization, 68°C; washes, 0.1 × SSC/0.5% SDS, 68°C; film exposure, 15 min. (d) Blot in (c) stripped and reprobed with a DNA fragment encoding part of the xanthomonadin gene of *X. campestris pv. campestris*. Hybridization, 63°C; washes, 0.2 × SSC/0.1% SDS, 63°C; film exposure, 3 h.

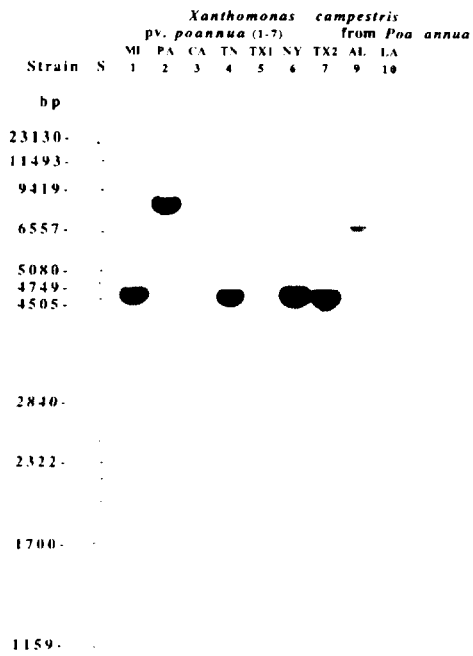
a



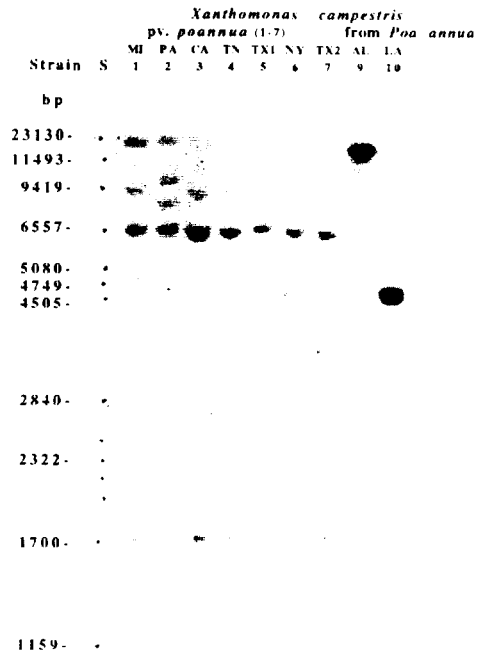
b

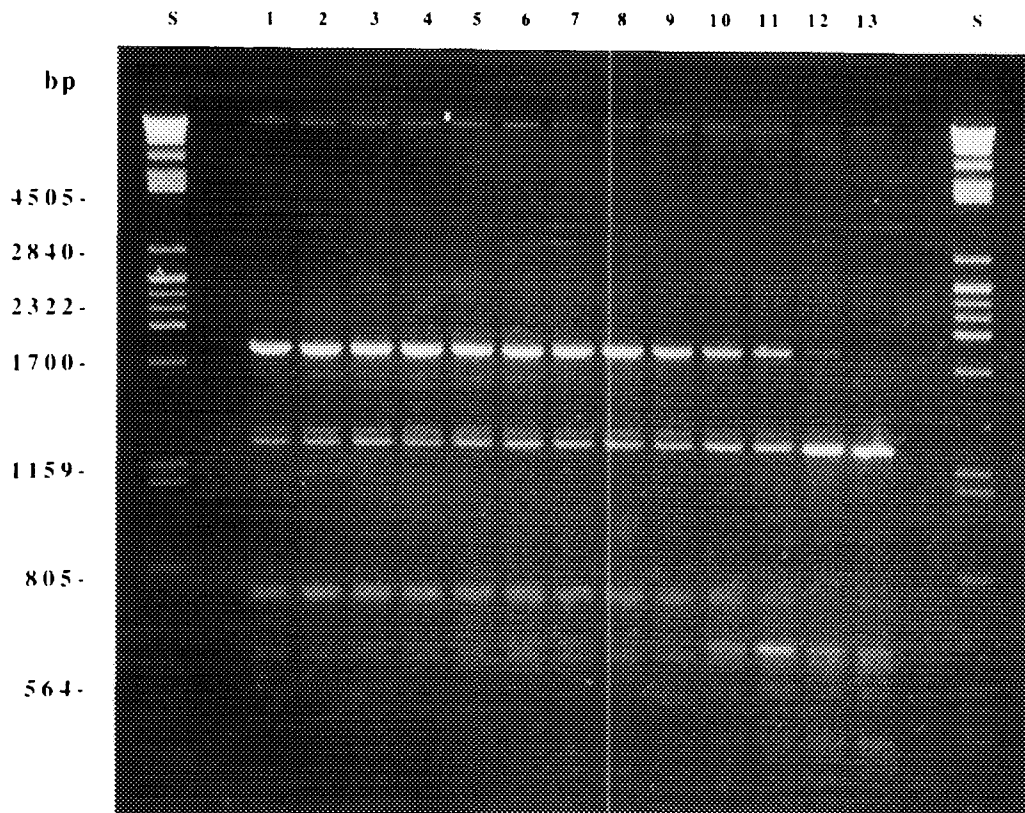


c



d





**FIG. 4.** Genomic DNA mixing experiment. DNA from *X.c. pv. poannua* (strain 1), which has a strong 1.8-kb band, was mixed in different proportions with DNA from *X.c. pv. poannua* (strain 3), which has a strong 1.2-kb band. Ethidium bromide-stained agarose gel shows RAPD products amplified under standard conditions (i.e., 25 ng template DNA total) using primer RWII. Lanes 1 through 13 contained 100, 99, 90, 80, 70, 60, 50, 40, 30, 20, 10, 1, and 0% DNA from strain 1, respectively, and 0, 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 99, and 100% DNA from strain 3, respectively. S, MW standards, a mixture of *Hind*III-digested and *Pst*I-digested  $\lambda$  DNA.

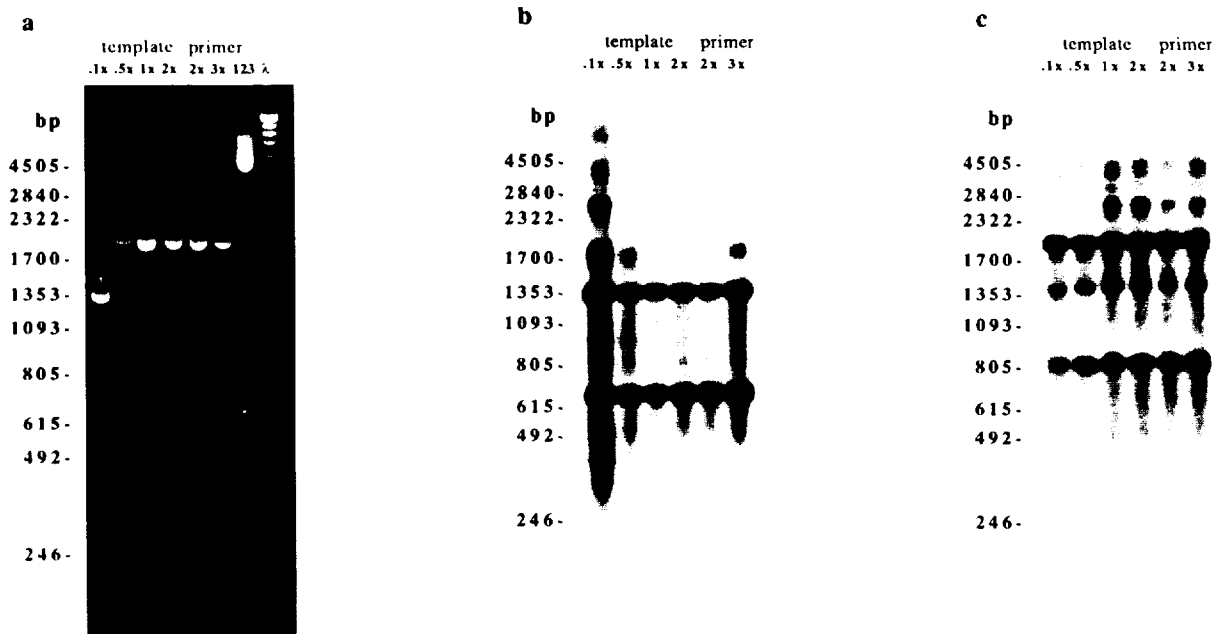
*X.c. pv. poannua* strains 1, 2, 4, 6, and 7 was greatly reduced compared to amplification in *X.c. pv. poannua* strains 3, 5, and 8. Heun and Helentjaris (1993) and Williams *et al.* (1993) pointed out that the formation of a given RAPD product is subject to competition. For example, Heun and Helentjaris (1993) demonstrated that amplification of a given RAPD product can depend upon the genetic background in which it is located, even though the primer binding site remains unaltered. We hypothesize that in strains with potential to produce both a 1.2 and 1.8-kb RAPD product (i.e., *X.c. pv. poannua* strains 1, 2, 4, 6, and 7), the 1.8-kb product is the preferred product because it outcompetes the 1.2-kb product.

This hypothesis was tested by means of a genomic DNA mixing experiment (Fig. 4), similar to the one carried out by Williams *et al.* (see Fig. 9A in Williams *et al.*, 1993). Genomic DNA from *X.c. pv. poannua* strain 1, which yields both a 1.8 and a 1.2-kb RAPD product, was mixed with DNA from *X.c. pv. poannua* strain 3, which yields only the 1.2-kb product. If the two products are equally favored, one would expect to see a linear increase in the 1.2-kb product with a corre-

sponding decrease in the 1.8-kb product as the ratio of *X.c. pv. poannua* strain 1 DNA to *X.c. pv. poannua* strain 3 DNA decreases. Furthermore, the relative intensities of the bands should be equivalent when each DNA template is present as 50% of the total. This is *not* what we observed. The intensity of the band representing the 1.8-kb product was equivalent to the intensity of the band representing the 1.2-kb product when the reaction mixture contained only 10% *X.c. pv. poannua* strain 1 DNA, and the 1.8-kb product was the dominant product when the percentage of *X.c. pv. poannua* strain 1 DNA reached 20%. These data support the hypothesis that the 1.8-kb RAPD product is favored in direct competition with the 1.2-kb product.

#### *Influence of Reaction Conditions on RAPD Profiles*

Many workers have observed a dependency of RAPD profiles on reaction conditions. For example, Ellsworth *et al.* (1993) showed that RAPD profiles were particularly prone to change at low template concentrations. We made similar observations. In the five *X.c. pv. poannua* strains with both a 1.8 and a 1.2-kb RAPD product, the relative amounts of the 1.8 and 1.2-kb



**FIG. 5.** Effect of template and primer concentration on RAPD products amplified from *X.c. pv. poannua*. Keeping all other PCR conditions constant, either template DNA from *X.c. pv. poannua* (strain 1) was varied from 2.5 to 50 ng ( $0.1\times$ – $2.0\times$  standard amount) or primer concentration was varied from 1.0 to 3.0  $\mu\text{M}$  ( $1.0\times$ – $3.0\times$ ). RAPD products were blotted and probed with either the pJRL100 or the pJRL200 insert. (a) Agarose gel of RAPD products showing shift of the major RAPD product from the 1.2-kb product at 2.5 ng template to the 1.8-kb product at higher template amounts. (b) Southern blot with the pJRL100 insert shows presence of the 1.2-kb product in all samples. (c) Southern blot with the pJRL200 insert shows presence of the 1.8-kb product in all samples. The preferential amplification of either the 1.2-kb product at low template concentration or the 1.8-kb product at high template concentration is reflected by the presence of higher molecular weight bands in the respective Southern blots. Sample: 0.5  $\mu\text{l}$ /25  $\mu\text{l}$  total reaction in each lane. Standards:  $\lambda$ , a mixture of *Hind*III-digested  $\lambda$  DNA and *Pst*I-digested  $\lambda$  DNA; 123, 123-bp ladder (Gibco BRL). Hybridization, 66°C; washes,  $0.1\times$  SSC/0.5% SDS, 68°C; film exposures, (b) 5 min; (c) 2 min.

products were dependent upon reaction conditions, in particular, template concentration (Fig. 5). A 10-fold decrease in template concentration led to a change of the major RAPD product in these strains from the 1.8-kb DNA to the 1.2-kb DNA product. Changing the primer concentration 2- or 3-fold did not produce major differences in the RAPD product profiles (Fig. 5).

One plausible explanation for the switch in the preferred RAPD product from the 1.8-kb product to the 1.2-kb product at very low template concentration is that the genomic template for the 1.8-kb product has better matched primer binding sites than the 1.2-kb product. Even if the primer binding site match is imperfect in the genomic DNA, in the PCR products the match becomes perfect. Under low initial template DNA conditions (few target DNA molecules), the proportion of template DNA molecules for the 1.2-kb product with perfect primer binding site matches becomes high early in the PCR. The presence of such templates would then allow the 1.2-kb product to be preferentially amplified over the 1.8-kb product, due either to kinetic or to thermodynamic considerations. However, an alternative explanation, that the primer binding site of the 1.2-kb product is different in *X.c. pv. poan-*

*nua* strains 1, 2, 4, 6, and 7 than in *X.c. pv. poannua* strains 3, 5, and 8, cannot be ruled out without further experimentation.

## DISCUSSION

### *RAPD Variation and Its Use in Phylogenetic Analysis*

Characterization of a set of RAPD products, obtained using a single primer to amplify DNA from 47 *Xanthomonas* strains, has provided information about the nature of the variation uncovered by the RAPD technique and how this measure of variation might best be used in comparative biology.

Phylogenetic analysis can be carried out using either a phenetic approach based on the analysis of overall similarity or a cladistic approach, in which relationships are based on or inferred from shared derived characters. It is beyond the scope of this paper to argue the relative merits of the two approaches, but for our own work, in which we wish to determine relationships between organisms, we prefer the cladistic approach. In phylogenetic analysis using a cladistic approach, hypotheses are proposed after analysis of characters that are either assumed or demonstrated to be homolo-

gous, independent, and variable (Swofford and Olsen, 1990). If these conditions are not met, the analysis will not yield valid results.

By direct examination of ethidium bromide-stained agarose gels, it was difficult to determine if the RAPD products obtained using the primer RWII to amplify DNA from 47 *X. campestris* strains were homologous and independent. For example, the 1.2-kb RAPD product from *X.c. pv. poannua* strains 1, 2, 4, 6, and 7 was often very faint or absent on agarose gels (Figs. 1a and 2a). Southern analysis, however, revealed that the 1.2-kb product was amplified in all *X.c. pv. poannua* strains (Fig. 2b), but at times to levels below the detection threshold for visualization by ethidium bromide staining. Although routine Southern analysis of all RAPD products would lessen the attractiveness of this technique, our results make it clear that in important instances such further efforts are essential if valid conclusions are to be reached.

A second problem arises when RAPD products of similar molecular weight are observed on gels but are not the products of amplification from homologous DNA segments. For example, the *X.c. pv. poannua* strains and the three strains of *X.c. pv. agrostis* all yield a RAPD product of 1.2 kb (Figs. 1 and 3b). These apparent homologies were invalid, however, since neither the RAPD products nor the genomic DNA of the three *X.c. pv. agrostis* strains hybridized to cloned 1.2-kb RAPD product (pJRL100) from *X.c. pv. poannua* (not shown). A possible solution to this problem would be to run longer gels in order to improve the resolution of DNA fragments. Alternatively, the RAPD products could be digested with a restriction endonuclease with a four-base recognition site and restricted RAPD products analyzed by gel electrophoresis. This second step would reveal which RAPD products shared restriction sites and were thus more likely to be homologous.

A third possible source of error arises when the presence or absence of some RAPD products is dependent upon the presence or absence of other RAPD products. This lack of independence was seen in our experiments and examined more closely using the data presented in Fig. 4. The presence of the 1.2-kb RAPD product from *X.c. pv. poannua* strains is apparently dependent on the absence of the 1.8-kb RAPD product, since when genomic DNA from *X.c. pv. poannua* strain 1 (produces strong 1.8-kb band) was mixed with an equal amount of DNA from *X.c. pv. poannua* strain 3 (produces 1.2-kb band and no 1.8-kb band), the 1.8-kb band predominated (Fig. 4, lane 6). Apparently, equal amounts of the two RAPD products were produced only when the strain 3 DNA was present in a ninefold excess over strain 1 DNA. These two RAPD products are clearly not synthesized in an independent manner and demonstrate the effect that genetic background has on the appearance of RAPD products, as documented by Heun and Helentjaris (1993). This lack of independence cannot easily be overcome technically, but the scoring of

many primers might possibly reduce this problem to an acceptable level.

A final difficulty encountered in our experiments was the presence of multiple related PCR products in a single reaction. The pJRL100 insert hybridized to at least two products and pJRL200 to at least three products in each hybridizing *X.c. pv. poannua* strain (Fig. 2). If single bands are used as characters, the presence of multiple related products could lead to an overestimation of between-strain similarities and differences. These bands could have arisen from multiple priming sites within the 1.2 and 1.8-kb DNA fragments or may represent alternative molecular forms of the RAPD products. One possible way to circumvent the problem of these multiple products would be to score the entire pattern as a single character or to score only a single band from each reaction.

The above discussion does not mean to imply that RAPD products have no place in phylogenetic studies. Numerous studies have been published recently using RAPD banding patterns to establish phenetic relationships (Kambhampati *et al.*, 1992; Grajal-Martin *et al.*, 1993; Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Ti-bayrenc *et al.*, 1993; Williams and St. Clair, 1993; Yang and Quiros, 1993). For workers performing such analyses, however, we suggest that some of the problems encountered in our experiments are not likely to be unique to our system and that care must be taken to ensure that the use of RAPD products as characters in phylogenetic studies produces valid results.

#### *Pathovar Specificity of pJRL100*

RFLP analysis, using cloned RAPD products as hybridization probes, is another potentially useful application. The RFLP patterns we obtained using RAPD products as probes were simple (Fig. 3), and assumptions of character homology are likely to be correct. In addition, fragment distributions within a sample should be informative. For example, the 1.2-kb RAPD product amplified from the *X.c. pv. poannua* strains is apparently unique to them and may serve as a diagnostic, pathovar-specific DNA marker. The pJRL100 insert hybridized only to the genomic DNA and RAPD products of the eight *X.c. pv. poannua* strains, and not to any of the other strains. Significantly, these eight *X.c. pv. poannua* strains originated from eight distinct geographical locations in seven states (Table 1). In addition, pJRL100 did not hybridize to the genomic DNA of the two nonpathogenic *X. campestris* strains that were isolated from *P. annua* L., while hybridization with the xanthomonadin probe (Fig. 3d) showed correct identification as *X. campestris*. DNA from *X.c. pv. poae*, whose host plant, *Poa trivialis* L., is more closely related to *P. annua* L. than the host plants of the other strains in the collection, also did not hybridize to pJRL100. However, definitive proof of pathovar specificity will require testing the pJRL100 insert with an expanded collection of bacterial strains.

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## REFERENCES

- Bradbury, J. F. (1984). *Xanthomonas* Dowson 1939. In "Bergey's Manual of Systematic Bacteriology" (N. R. Krieg and J. G. Holt, Eds.), Vol. 1, pp. 199–210, Williams & Wilkins, Baltimore.
- Caetano-Anolles, G., Bassam, B. J., and Gresshoff, P. M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* **9**: 553–556.
- Crowhurst, R. N., Hawthorne, B. T., Rikkerink, E. H. A., and Templeton, M. D. (1991). Differentiation of *Fusarium solani* f. sp. *curbitae* races 1 and 2 by random amplification of polymorphic DNA. *Curr. Genet.* **20**: 391–396.
- Ellsworth, D. L., Rittenhouse, K. D., and Honeycutt, R. L. (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. *BioTechniques* **14**: 214–217.
- Feinberg, A., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**: 266–267.
- Goodwin, P. H., and Annis, S. L. (1991). Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. *Appl. Environ. Microbiol.* **57**: 2482–2486.
- Grajal-Martin, M. J., Simon, C. J., and Muehlbauer, F. J. (1993). Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f. sp. *pisi*. *Phytopathology* **83**: 612–614.
- Hadrys, H., Balick, M., and Schierwater, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* **1**: 55–63.
- Hedrick, P. (1992). Shooting the RAPDs. *Nature* **355**: 679–680.
- Heun, M., and Helentjaris, T. (1993). Inheritance of RAPDs in F1 hybrids of corn. *Theor. Appl. Genet.* **85**: 961–968.
- Hillis, D. M., and Moritz, C. (1990). "Molecular Systematics," Sinauer Associates, Sunderland, MA.
- Joshi, C. P., and Nguyen, H. T. (1993). Application of the random amplified polymorphic DNA technique for the detection of polymorphism among wild and cultivated tetraploid wheats. *Genome* **36**: 602–609.
- Kambhampati, S., Black, W. C., IV, and Rai, K. (1992). Random amplified polymorphic DNA of mosquito species and populations (Diptera: Culicidae): Techniques, statistical analysis, and applications. *J. Med. Entomol.* **29**: 939–945.
- Kazan, K., Manners, J. M., and Cameron, D. F. (1993). Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. *Genome* **36**: 43–49.
- Klein-Lankhorst, R. M., Vermunt, A., Weide, R., Liharska, T., and Zabel, P. (1991). Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). *Theor. Appl. Genet.* **83**: 108–114.
- Landry, B. S., Dextraze, L., and Boivin, G. (1993). Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome* **36**: 580–587.
- Martin, G. B., Williams, J. G. K., and Tanksley, S. D. (1991). Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc. Nat. Acad. Sci. USA* **88**: 2336–2340.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.* **149**: 114–122.
- Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Nat. Acad. Sci. USA* **88**: 9828–9832.
- Paran, I., Kesseli, R. V., and Michelmore, R. W. (1991). Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines. *Genome* **3**: 1021–1027.
- Poplawsky, A. R., Kawalek, M. D., and Schaad, N. W. (1993). A xanthomonadin-encoding gene cluster for the identification of pathogens of *Xanthomonas campestris*. *Mol. Plant-Microbe Interact.* **6**: 545–552.
- Riedy, M. F., Hamilton, W. J., and Aquadro, C. F. (1992). Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. *Nucleic Acids Res.* **20**: 918.
- Roberts, D., Evans, S., Detweiler, R., and Vargas, J. M., Jr. Description of *Xanthomonas campestris* pathovar poannua, a narrow-spectrum wilt pathogen of *Poa annua* L., in preparation.
- Sambrook, J. S., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Swofford, D. L., and Olsen, G. J. (1984). Phylogeny reconstruction. In "Molecular Systematics" (D. M. Hillis and C. Moritz, Eds.), pp. 411–501, Sinauer Associates, Sunderland, MA.
- Tibayrenc, M., Neubauer, K., Barnable, C., Guerrini, F., Skarecky, D., and Ayala, F. J. (1993). Genetic characterization of six parasitic protozoa: Parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proc. Natl. Acad. Sci. USA* **90**: 1335–1339.
- Vidaver, A. (1967). Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic *Pseudomonads*: Effect of the carbon source. *Appl. Microbiol.* **15**: 1523–1524.
- Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213–7218.
- Welsh, J., Pretzman, C., Postic, D., Saint Girons, I., Baranton, G., and McClelland, M. (1992). Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. *Int. J. Syst. Bacteriol.* **42**: 370–377.
- Wiley, E. O. (1981). "Phylogenetics: The Theory and Practice of Phylogenetic Systematics," Wiley-Interscience, New York.
- Williams, C. E., and St. Clair, D. A. (1993). Phenetic relationships and levels of variability detected by restriction fragment length polymorphism and random amplified polymorphic DNA analysis of cultivated and wild accessions of *Lycopersicon esculentum*. *Genome* **36**: 619–630.
- Williams, J. G. K., Hanafey, M. K., Rafalski, J. A., and Tingey, S. V. (1993). Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol.* **218**: 704–740.
- Williams, J., Kubelik, A., Livak, K., Rafalski, J., and Tingey, S. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531–6535.
- Yang, X., and Quiros, C. (1993). Identification and classification of celery cultivars with RAPD markers. *Theoret. Appl. Genet.* **86**: 205–212.