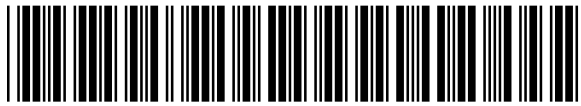

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A Repetitive DNA Sequence Differentiates *Xanthomonas campestris* pv. *oryzae* from Other Pathovars of *X. campestris*

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Two repetitive DNA sequences were detected in the genome of *Xanthomonas campestris* pv. *oryzae*. An *Eco*RI-*Hin*dIII restriction fragment (2.4 kilobases) containing one highly repetitive sequence was cloned (pJEL101). Approximately 81 copies of the repetitive sequence in pJEL101 were dispersed throughout the genome as determined by quantitative filter hybridizations. Repetitive sequences related to pJEL101 were detected in all strains of *X. c.* pv. *oryzae* from different geographic areas. Strains representing five of 25 other *X. campestris* pathovars appeared to contain multiple copies of a related element. Only *X. c.* pv. *oryzicola*, another rice pathogen, contained a relatively high copy number of pJEL101 sequences compared to *X. c.* pv. *oryzae*, although the hybridization banding pattern was different. The pJEL101 sequence is a useful probe for pathovar identification as well as strain and population analyses.

Additional keywords: restriction fragment length polymorphism.

Xanthomonas campestris pv. *oryzae* is the causal agent of bacterial leaf blight, a major rice disease prevalent throughout Asia. Traditionally, bacterial leaf blight is controlled by planting resistant rice cultivars. However, over time, virulent strains of *X. c.* pv. *oryzae* have been detected on the resistant cultivars (Mew 1987). In an effort to understand pathogenic variability, restriction fragment length polymorphism (RFLP) analysis of the genetic relationships of *X. c.* pv. *oryzae* strains was initiated. RFLP analysis has been used to study genetic relationships of bacterial genera, species, and pathovars (Cook *et al.* 1989; Denny 1988; Denny *et al.* 1988; Gabriel *et al.* 1988; Lazo *et al.* 1987). Our initial approach, however, was confounded because complex hybridization patterns, suggestive of repetitive DNA sequences, were observed when genomic cosmid clones were used as probes (Leach *et al.* 1988).

Although there are few reports of repetitive elements in *Xanthomonas* spp. (Bonas *et al.* 1989; Kearney and Staskawicz 1990), repetitive DNA sequences have been found in other bacterial genomes. Such sequences include the 11-base pair (bp) sequence involved in the uptake of DNA by *Haemophilus influenzae* (Smith *et al.* 1981), the 35-bp repetitive extragenic palindromic sequence of *Escherichia coli* and *Salmonella typhimurium* (Higgins *et al.* 1982; Stern *et al.* 1984), and transposable elements (insertion sequences, transposons, or transposing bacteriophages) ranging in size from 700 to greater than 10,000 bp (Appelbaum *et al.* 1985; Comai and Kosuge 1983; Flores *et al.* 1987; Iida *et al.* 1983; Kaluza *et al.* 1985; Kleckner 1981; McLafferty *et al.* 1988; Mogen and Oleson 1987; Ruvkun *et al.* 1982). Copy numbers of the repeated sequences vary from two to greater than 40 for insertion sequences (Iida *et al.* 1983) and up to 1,000 for the repetitive palindromic sequence (Stern *et al.* 1984). Although the function of most repetitive elements is not clear, some may be involved in adaptation by facilitating DNA rearrangements and altering gene expression (Iida *et al.* 1983; Kearney *et al.* 1988; Kleckner 1981; Ruvkun *et al.* 1982; Sapienza *et al.* 1982; Scordilis *et al.* 1987; Stern *et al.* 1984; Szabo and Mills 1984).

We report here the presence of highly repetitive DNA sequences (elements) in *X. c.* pv. *oryzae*. In addition, we describe the distribution of one element among strains of *X. c.* pv. *oryzae* and pathovars of *X. campestris* and the usefulness of that element as a probe for diagnosis of *X. c.* pv. *oryzae*.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmids used in this study are listed in Table 1.

The strains of *X. c.* pv. *oryzae* provided by A. Alvarez, University of Hawaii, Honolulu, were AXO-R3 from Australia; BXO-13 from Burma; TXO-R7 from Thailand; SLXO-CL1 from Sri Lanka; IXO-R33 from India; and JXO-R-48, JXO-H75304, JXO-T7133, JXO-H75373, and JXO-T7174 from Japan. Strains from Australia provided by L. Diatloff, Agricultural Research Laboratories, Indooroopilly, Queensland, Australia, included AXO-2028, AXO-2031, and AXO-2052. Strains from Colombia provided by J. Lozano, Centro Internacional de Agricultura Tropical, Cali, Colombia, were CXO-1185 and CXO-1186. Strains from India provided by K. Gupta, Punjab Agricultural University, Ludhiana, India, were IXO-1 and IXO-10. Strain N163 from Nepal was provided by T. Adhikari, National Rice Improvement Program, Kathmandu, Nepal. Strain IXO-B1 originating in Bangladesh was from this laboratory. Strains provided by C. Gonzalez, Texas A. & M. University, College Station,

originated in the United States and included UXO-87-17 from Louisiana and UXO-X-1-7, UXO-X-1-8, and UXO-X-1-1 from Texas; C. Rush, Louisiana State University and A. & M. C., Baton Rouge, provided UXO-X8-1A from Louisiana. Strains provided by T. Mew, The International Rice Research Institute, Los Baños, the Philippines, originated in the Philippines and included PXO61 from Lucban, Quezon, PXO86^{Rif} from Los Baños, Laguna, PXO79 from Davao, PXO70 from Palawan, PXO112 from Banaue, Ifugao, and PXO124 from Los Baños, Luzon.

Other bacterial strains provided by the American Type Culture Collection, Rockville, MD, were *X. c.* pv. *glycines* 17915, *X. c.* pv. *phaseoli* 9563, and *X. c.* pv. *pruni* 19316. Strains from the International Collection of Phytopathogenic Bacteria, University of California, Davis, were *X. c.* pv. *begoniae* XB110, *X. c.* pv. *glycines* NZ2336, and *X. c.* pv. *holcicola* XH112. Strains from the Department of Plant Pathology, Kansas State University, Manhattan, included *E. coli* TB1; *X. c.* pv. *alfalfae* KX-1, FX1-1, and FX2-1; *X. c.* pv. *campestris* KXCC1; *X. c.* pv. *holcicola* 86; *X. c.* pv. *malvacearum* 535 and 536; *X. c.* pv. *pelargonii* NE1985; *X. c.* pv. *secalis* XT104; *X. c.* pv. *sojense* 109; and *X. c.* pv. *translucens* XT115, XT110, and XT103. A. Vidaver, University of Nebraska, Lincoln, provided *X. c.* pv. *sojense* UNL4455 and *X. c.* pv. *translucens* NEB101. C. Gonzalez provided a *Xanthomonas* spp. that was isolated from, but not pathogenic to rice. Strains provided by C. Stevens, Michigan State University, East Lansing, included *X. c.* pv. *pelargonii* X-7, X-5, and X-1. Strains provided by R. Gilbertson, University of Wisconsin, Madison, were *X. c.* pv. *phaseoli* WT8 and CNF31 and *X. c.* pv. *phaseoli* var. *fuscans* Bat67 and Xcpf8. R. Stall, University of Florida, Gainesville, provided *X. c.* pv. *cucurbitae* Xccu1, *X. c.* pv. *gummisudans* Xgu1, and *X. c.* pv. *vesicatoria* 81-23 and 82-8. Strains from the National Collection of Plant Pathogenic Bacteria, Hertfordshire, England, were *X. c.* pv. *campestris* 528; *X. c.* pv. *carotae* 1422; *X. c.* pv. *cerealis* 1836 and 1943; *X. c.* pv. *cucurbitae* 2597; *X. c.* pv. *graminis* 2700; *X. c.* pv. *phleipratensis* 5744; *X. c.* pv. *pisi* 762; *X. c.* pv. *undulosa* 1945; *X. c.* pv. *vasculorum* 206 and 1326; *X. c.* pv. *zinnae* 2439; and *X. albilineans* 2969. Strains from the Plant Diseases Division Culture Collection, Auckland, New Zealand included *X. c.* pv. *hordei* 5735, *X. c.* pv. *phaseoli* 2729, *X. c.* pv. *phleipratensis* 1839, *X. c.* pv. *secalis* 5749, *X. c.* pv. *translucens* 5752, *X. c.* pv. *undulosa* 5755, and *X. fragariae* 10056. Strains provided by T. Mew, The International Rice Research Institute, were *X. c.* pv. *oryzicola* BLS101, BLS175, BLS179, BLS256, BLS288, BLS292, BLS290, BLS298, BLS295, and BLS303. J. Lozano provided *Pseudomonas* spp. strains 1173 and 1171.

Table 1. Plasmids used in this study

Designation	Description[a]	Source or reference
pLAFR3	IncP, Tc^r, Mob$^+$, *cos*	Staskawicz *et al.* 1987
pSa747	IncW, Km^r, Mob$^+$, *cos*	Tait *et al.* 1983
pUC18	ColE1 replicon, Ap^r	Norrander *et al.* 1983
pBluescript KS+	ColE1 replicon, Ap^r	Stratagene, La Jolla, CA
pL86a	32-kb genomic clone from PXO86^{Rif} in pLAFR3	This study
pS86b	41-kb genomic clone from PXO86^{Rif} in pSa747	This study
pL86C	18-kb genomic clone from PXO86^{Rif} in pLAFR3	This study
pS86d	33-kb genomic clone from PXO86^{Rif} in pSa747	This study
pL86e	34-kb genomic clone from PXO86^{Rif} in pLAFR3	This study
pJEL101	2.4-kb *Eco*RI-*Hin*dIII fragment from pL86a, contains repetitive sequence	This study
pBS101	2.4-kb *Eco*RI-*Hin*dIII fragment from pJEL101 in pBluescript KS+	This study
pBS8-4	2.5-kb *Eco*RI fragment in pBluescript KS+, contains single copy *avr10* gene	Kelemu and Leach 1990

[a] Tc^r, Km^r, and Ap^r indicate resistance to tetracycline, kanamycin, and ampicillin, respectively; and kb, kilobase.

Culture conditions. Strains of *X. c.* pv. *oryzae* were maintained on peptone-sucrose agar (Tsuchiya *et al.* 1982) at 28° C; *E. coli* was maintained on Luria-Bertani agar (Miller 1972) at 37° C; and all other bacteria were maintained on nutrient agar (Difco Laboratories, Detroit, MI) at 28° C.

DNA isolation. Bacteria were grown in 15 ml of nutrient broth (Difco) for 15 hr at 28° C on a rotary shaker (200 rpm). Genomic DNA was extracted by a modification of the procedure of Owen and Borman (1987). Bacterial cells were pelleted by centrifugation (13,776 × *g*); resuspended in a mixture of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM Na_2EDTA, 2 mg/ml freshly mixed lysozyme (total volume, 3.3 ml); and incubated at room temperature for 20 min. To each DNA sample, sodium dodecyl sulfate (SDS; 167 μl of a 10% solution) was added. After incubation for 10 min at 50° C, 134 μl of RNase A (2.5 mg/ml in 10 mM Tris-HCl, pH 7.6) was added, and the mixture was incubated at 37° C for 1 hr. Then, 170 μl of 0.5 M Na_2EDTA was added, and the mixture was incubated for 10 min at 50° C. Proteinase K (50 μl of a 5 mg/ml solution in 10 mM Tris-HCl, pH 7.0) was added, and the mixture was incubated for 3–12 hr at 37° C. Each sample was then extracted with two equal volumes of phenol saturated with 0.1 M Tris-HCl (pH 8.0), one volume of a 1:1 phenol and chloroform-isoamyl alcohol (24:1) mixture, and one volume of chloroform-isoamyl alcohol. The DNA was precipitated with ethanol and redissolved in 500 μl of 10 mM Tris, pH 8.0, and 1 mM Na_2EDTA. DNA concentrations were estimated spectrophotometrically.

Cloning of DNA fragments. A cosmid library of strain PXO86^{Rif} DNA constructed by ligation of partially digested *Eco*RI fragments into pSa747 was described previously (Kelemu and Leach 1990). A second library of *Sau*3A partial fragments was constructed from DNA of strain PXO86^{Rif} in pLAFR3 as described by Swanson *et al.* (1988). Subcloning procedures and plasmid analysis and isolation procedures were as described in Maniatis *et al.* (1982).

RFLP analysis. Bacterial DNA (10 μg) was digested to completion with *Eco*RI (2 units per microgram of DNA)

at 37° C for 2–3 hr in buffers provided by Bethesda Research Laboratories (BRL, Gaithersburg, MD) or Promega (Madison, WI). The DNA fragments (2–3 μg per well) were separated in 0.7% agarose gels immersed in Tris-borate buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM Na_2EDTA, pH 8.0) by electrophoresis. A 1-kilobase (kb) ladder (BRL) was included in gels as a size standard.

Transfer of DNA fragments from agarose gels onto nylon membranes, Southern hybridizations, and high-stringency washes were done as described by the manufacturer of the GeneScreen Plus membrane (Du Pont Co., Wilmington, DE). Plasmid preparations were labeled with [^{32}P]CTP using a nick translation kit (BRL). Blots were prehybridized at 65° C for 3–4 hr in a solution composed of 0.1% SDS, 50 mM sodium phosphate buffer (PB, pH 7.0), 1.0 M NaCl, and 300 μg/ml denatured salmon sperm DNA. For hybridization, denatured labeled probe DNA (10^6 cpm/ml) was added directly to the prehybridization solution, and the blot was incubated at 65° C for 18 hr. After hybridization, the blot was washed three times at 65° C in 2× SSC (20× SSC contains 3 M NaCl and 0.3 M Na_3-citrate) containing 0.1% SDS and 5 mM PB and then three times in 0.5× SSC containing 0.1% SDS and 3 mM PB. Autoradiographic exposures were at −80° C using Cronex film (Du Pont) and a Du Pont Cronex Hi-Plus intensifying screen.

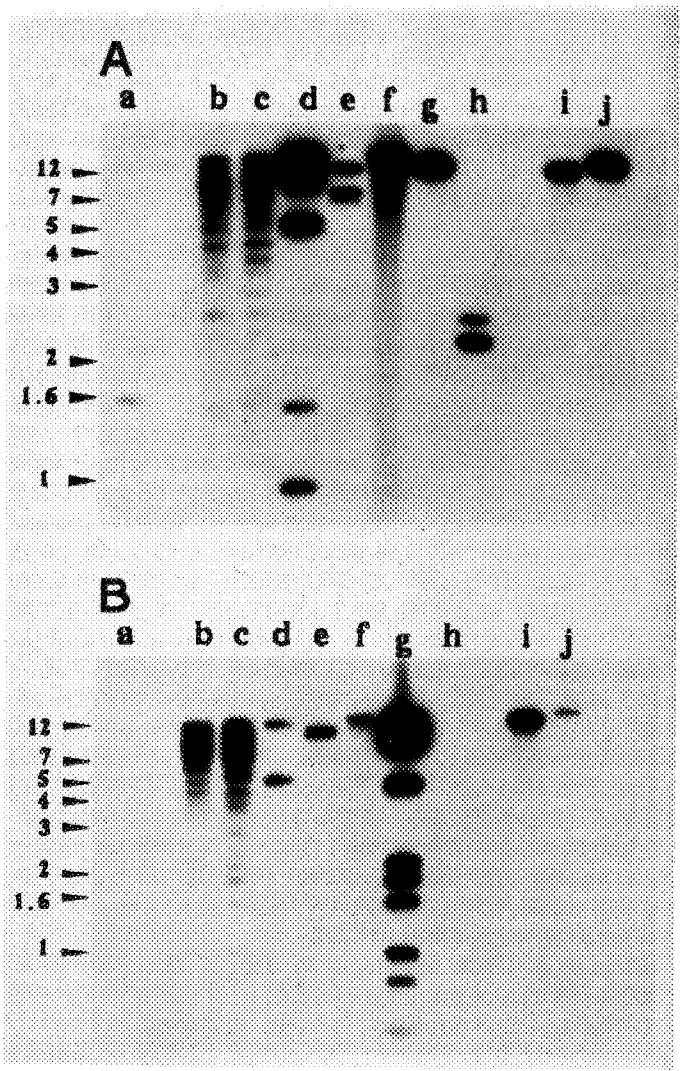

Fig. 1. Autoradiographs of identical Southern blots after hybridization with ^{32}P-labeled clones pL86a (**A**) and pS86d (**B**). Lane a, 1-kilobase ladder; lanes b and c, genomic DNA of *Xanthomonas campestris* pv. *oryzae* PXO61 and PXO86^{Rif}, respectively; lane d, pL86a; lane e, pS86b; lane f, pL86c; lane g, pS86d; lane h, pJEL101; lane i, pSa747; and lane j, pLAFR3. DNA samples were digested with *Eco*RI before electrophoresis and transfer. pJEL101 was digested with *Eco*RI and *Hin*dIII.

To compare strains of *X. c.* pv. *oryzae* and *X. c.* pv. *oryzicola*, the presence or absence of hybridization bands corresponding to DNA fragments of 6 kb or less was scored for each strain. Comparisons were only made within individual blots. The proportion of mismatched fragments was calculated for all pairwise combinations of strains as described by Denny *et al.* (1988).

Deletion analysis of the repetitive sequence in pJEL101. The 2.4-kb *Eco*RI-*Hin*dIII fragment contained in pJEL101 was cloned into pBluescript KS+ (Stratagene, La Jolla, CA) to construct pBS101 and subjected to deletion mutagenesis by digestion with exonuclease III as described by Ausubel *et al.* (1989). To visualize the presence of the repetitive sequences, deleted plasmids were digested with *Bam*HI (to linearize plasmids containing deletions from the *Hin*dIII side of the insert) or *Hin*dIII (to linearize plasmids containing deletions from the *Eco*RI side of the insert). Linearized plasmids were then separated by electrophoresis, transferred to nylon membranes, and probed with ^{32}P-labeled pS86b (Table 1).

Quantitative slot blot. The copy number of the repetitive DNA element in the genome of *X. c.* pv. *oryzae* was estimated by a modification of the quantitative dot blot procedure described by Pruitt and Meyerowitz (1986). DNA from *X. c.* pv. *oryzae* was diluted with salmon sperm DNA such that the total DNA concentration remained at 10 μg/ml. A 50-μl sample from each dilution was sheared and denatured by adding 50 μl of 0.6 M NaOH and heating at 50° C for 20 min. The mixture was then chilled on ice. Immediately before filtering, 400 μl of 20× SSC with 75 mM HCl was added to each tube, and 200-μl samples were then filtered slowly (15–30 sec per 100-μl sample) through a nitrocellulose membrane (Bio-Dot blotting media, Bio-Rad Laboratories, Richmond, CA) equilibrated in 20× SSC using a Bio-Slot microfiltration apparatus (Bio-Rad). The wells were each rinsed with 20× SSC. The filter was cut into strips containing 24 slots each (from each of two dilution series) and baked for 4 hr at 80° C in a vacuum oven. Hybridizations and autoradiography were as described above. The intensity of hybridization was measured by scanning densitometry.

Identical sets of membranes were hybridized with a clone in pBluescript KS+ containing a 2.5-kb single copy sequence (pBS8-4, Table 1) or with the clone containing the repetitive sequence (pJEL101). To obtain relative copy numbers of complementary sequences for each probe per genome, values from densitometric scans for duplicate dilution series were averaged and plotted against the quantity of genomic DNA on the filter. Lines were fitted by linear regression analysis, and the slopes were calculated from the linear portion of the curves. The number of copies

per genome was determined using the following equation:

copy no./genome = slope a/slope b
· specific activity b/specific activity a
· mol. wt. insert of b/mol. wt. insert of a

where a = probe pJEL101 and b = probe pBS8-4. Copy numbers are the average values of two experiments.

RESULTS

Identification of repetitive DNA sequences. Initially, randomly selected clones from genomic libraries of *X. c.* pv. *oryzae* were used as hybridization probes in Southern blots of *Eco*RI-digested DNA from *X. c.* pv. *oryzae*. For comparative purposes, the blots (shown in Fig. 1) were repeated to include a subclone of one repetitive sequence (pJEL101, Table 1, described in more detail below). The vectors (pLAFR3 or pSa747) hybridized with each other but not with genomic DNA from *X. c.* pv. *oryzae* (data not shown). Many of the cosmid clones (data are presented for two examples, pL86a and pS86d [Table 1]) hybridized to more fragments in digests of total genomic DNA from *X. c.* pv. *oryzae* than expected from the complexity of the individual clones. Clone pL86a contained six *Eco*RI fragments (sizes 25.0, 11.0, 11.0, 5.5, 1.6, and 1.0 kb, Fig. 1A, lane d). However, numerous bands were detected in total genomic DNA (Fig. 1A, lanes b and c). Clone pS86d contained nine *Eco*RI fragments (sizes 15.0, 11.4, 9.2, 4.9, 2.3, 1.9, 1.6, 1.0, and 0.8 kb, Fig. 1B, lane g) and also provided a complex band pattern with genomic DNA (Fig. 1B, lanes b and c). The hybridization patterns from both pL86a and pS86d were more complicated than would be expected for partial digestion of the genomic DNA. In addition, probes of single copy sequences (for example, pBS8-4, Table 1) yielded the expected single band patterns using the same DNA preparations (data not shown). Thus, the results were consistent with the presence of one or more repetitive sequences in the two genomic clones.

Clone pL86a hybridized with three *Eco*RI fragments in pS86b (Fig. 1A, lane e). The three hybridizing fragments represented vector pSa747 and two fragments of genomic origin (Fig. 1A, lane e). Since pS86b was generated by ligation of partial *Eco*RI fragments into pSa747, digestion of the clone yielded one vector fragment. Similarly, pL86a hybridized with the pSa747 fragment of pS86d (Fig. 1A, lane g). Probe pS86d hybridized with two bands of pL86a that contained the two vector arms of pLAFR3 (23.0 and 5.5 kb; Fig. 1B, lane d). Hybridization of pS86d with pS86b was due to the common vector fragment of pSa747 (Fig. 1B, lane e). Probe pS86d did not hybridize to clone pJEL101 containing the repetitive sequence (Fig. 1B, lane h). Since pS86d did not hybridize with genomic sequences in pS86b (Fig. 1B, lane e) and pL86a did not hybridize to genomic fragments from pS86d (Fig. 1A, lane g), we determined that pL86a and pS86d contained different repetitive elements.

Isolation of a *X. c.* pv. *oryzae* repetitive element. The clone pL86a was digested with *Eco*RI and *Hin*dIII, transferred to nylon membranes, and probed with pL86e (pL86e [Table 1], when used to probe genomic DNA, resulted in a pattern similar to that revealed by pL86a). A 2.4-kb *Eco*RI-*Hin*dIII fragment, which contained the region of hybridization, was identified and subcloned into pUC18. When the subclone (pJEL101) was used as a probe of total genomic DNA from two strains of *X. c.* pv. *oryzae*, a complex signal pattern similar to the pattern obtained with pL86a was observed (compare lanes b and c of Figs. 1A and 2). The genomic fragments in pS86b that were detected with pL86a were also detected with pJEL101 (Fig. 2, lane e). Clone pJEL101 also hybridized with pLAFR3 (Fig. 2, lane j), presumably through sequences shared between pUC18 and pLAFR3 since pLAFR3 contains the *Hae*II fragment of pUC18 (Staskawicz *et al.* 1987). The pUC18 vector does not hybridize with genomic DNA from *X. c.* pv. *oryzae* (data not shown). Clone pJEL101 did not hybridize to the pSa747 sequences (Fig. 2, lane i) or to any genomic fragments of pS86d (Fig. 2, lane g). Only two bands were detected in pL86a with pJEL101 (Fig. 2, lane d), and these were the same bands that hybridized with pS86d (Fig. 1B, lane d), indicating that the repetitive element was near the vector cloning site. Only the 5.4-kb fragment from pL86a hybridized with the *Eco*RI-*Hin*dIII fragment isolated from pJEL101 (data not shown).

A further test to confirm the difference of the element(s) in pJEL101 and pS86d was performed by probing identical genomic DNA filters with each clone. DNA from strain PXO86^{Rif} was digested with *Bam*HI, *Eco*RI, and *Hin*dIII and probed with pJEL101 and pS86d. The lanes representing the same enzyme digest were then aligned to compare the patterns from each probe. Where individual bands could be distinguished (bands less than 6 kb, Fig. 3), the majority of bands detected with both probes were unique in each of the three enzyme treatments.

To further characterize the distribution of the pJEL101 repetitive sequence in the genome of *X. c.* pv. *oryzae*, the clone was used as a probe against plasmid DNA from *X. c.* pv. *oryzae* and randomly selected cosmid clones of genomic DNA from *X. c.* pv. *oryzae*. The subclone did not hybridize with plasmid pPXO112 from strain PXO112 or plasmid pPXO124 from strain PXO124 (data not shown).

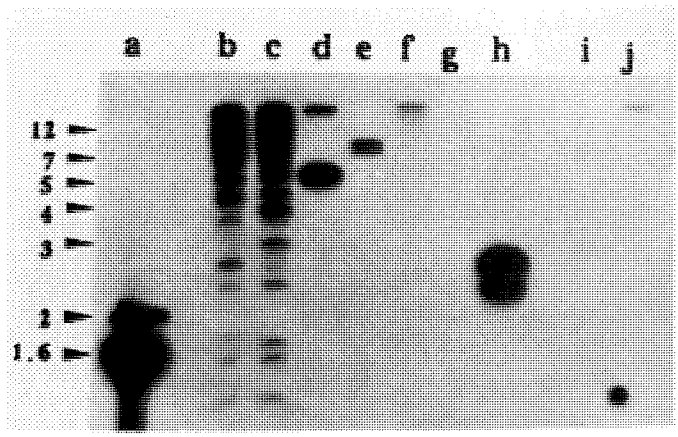

Fig. 2. Southern blot analysis of *Xanthomonas campestris* pv. *oryzae* total DNA and genomic clones with ^{32}P-labeled pJEL101. Lane a, 1-kilobase ladder; lanes b and c, genomic DNA from strains PXO61 and PXO86^{Rif} of *X. c.* pv. *oryzae*, respectively; lane d, pL86a; lane e, pS86b; lane f, pL86c; lane g, pS86d; lane h, pJEL101; lane i, pSa747; and lane j, pLAFR3. DNA samples were digested with *Eco*RI before electrophoresis and transfer. pJEL101 was digested with *Eco*RI and *Hin*dIII.

On the other hand, pJEL101 hybridized with 20 separate *Eco*RI-*Hin*dIII fragments in 17 of 20 clones tested from a PXO86^{Rif} genomic library in pSa747 (data not shown). The 20 cosmid clones were considered random because restriction analysis revealed different DNA fragmentation (data not shown) and characterization of the pSa747 library revealed no bias toward any particular clones (Kelemu and Leach 1990).

Radiolabeled pS86b, which also contained sequences that hybridized to the repetitive element, was used to probe a series of deletion derivatives generated from the *Eco*RI-*Hin*dIII fragment of pJEL101 (Fig. 4). Derivatives lacking about 1 kb from the *Eco*RI side of the fragment did not hybridize with pS86b, indicating that the repetitive element contained in pJEL101 was located near the *Eco*RI site and was approximately 1 kb (Fig. 4, lanes l and m). Since all deletions starting from the *Hin*dIII side of the *Eco*RI-*Hin*dIII fragment hybridized with pS86b (Fig. 4, lanes a–f), we could not determine if the *Eco*RI site was contained within the repetitive element or if the element ended just upstream of the *Eco*RI site.

An estimate of the sequence copy number represented by the repetitive element in pJEL101 was obtained from quantitative filter hybridization experiments. Filters containing serial dilutions of genomic DNA from *X. c.* pv. *oryzae* were treated separately with radioactively labeled pJEL101 and a single copy sequence from *X. c.* pv. *oryzae*, pBS8-4 (Table 1). After correction for the specific activity and length of hybridizing DNA of the different probes, the amount of hybridization of genomic DNA to pJEL101 and pBS8-4 was compared. Assuming the repetitive element was 1.0 kb (from deletion data), we determined that approximately 81 copies were present in the genome of *X. c.* pv. *oryzae*.

Distribution of the pJEL101 sequence in the genus *Xanthomonas*. To assess the use of the pJEL101 repetitive sequence as a diagnostic tool, the presence or absence of the sequence in 30 different strains of *X. c.* pv. *oryzae*, 25 other pathovars of *X. campestris*, and two different species of *Xanthomonas* (*X. albilineans* and *X. fragariae*) was determined. Hybridizations were performed with genomic DNA from strains of *X. c.* pv. *oryzae* obtained from diverse geographic origins. With the exception of strains from the United States, 30 or more bands from *Eco*RI-digested, total DNA from strains of *X. c.* pv. *oryzae* could be distinguished after hybridization with pJEL101 (Fig. 5, lanes a, b, and g–o). Although signal differences were evident, the patterns were similar in their complexity, and numerous common subgroups of bands (band complexes) were apparent. Strains from the United States also contained a repetitive element (Fig. 5, lanes c, e, and f). However, hybridization of DNA from strain UXO-X-1-7 from Texas yielded only 11 bands (Fig. 5, lane c). The DNA of strains from Louisiana contained only nine hybridizing fragments and had a pattern different from the UXO-X-1-7 pattern (compare lanes c, e,

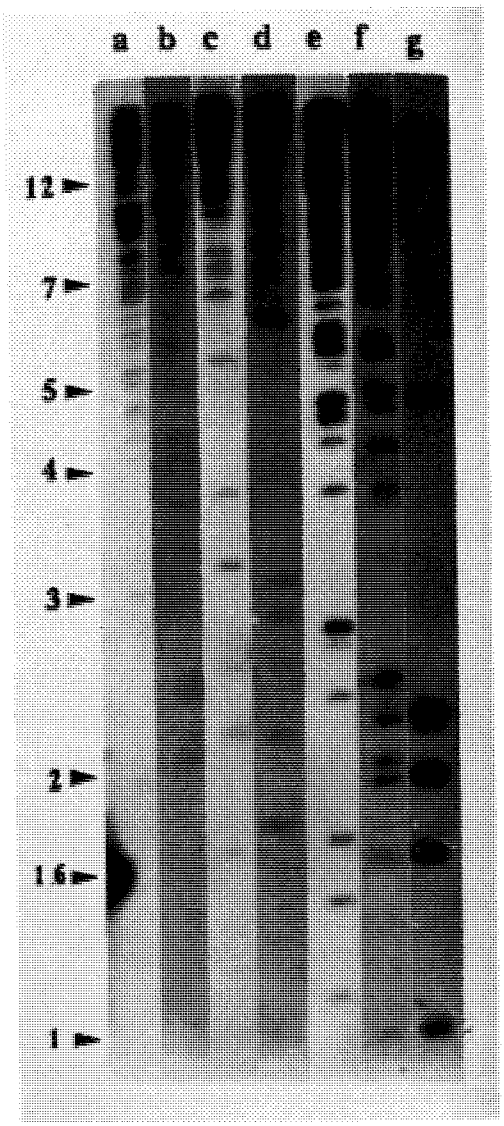

Fig. 3. Southern blot analysis of total *Xanthomonas campestris* pv. *oryzae* PXO86^{Rif} DNA with two repetitive sequences. DNA was digested with *Hin*dIII (lanes a and b), *Bam*HI (lanes c and d), and *Eco*RI (lanes e and f). Lane g, pS86d digested with *Eco*RI. Blots were probed with ^{32}P-labeled pJEL101 (lanes a, c, and e) and pS86d (lanes b, d, f, and g).

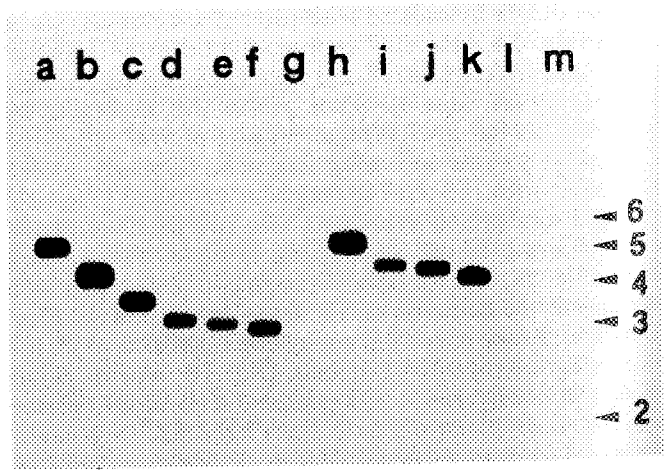

Fig. 4. Deletion analysis of the repetitive DNA element in the 2.4-kilobase *Eco*RI-*Hin*dIII fragment of pJEL101 (recloned as pBS101). Plasmids were sequentially deleted from the *Hin*dIII (lanes a–f) or *Eco*RI (lanes h–m) side of the insert by digestion with exonuclease III. Derivative plasmids were linearized by digestion with *Bam*HI (lanes a–f) or *Hin*dIII (lanes h–m). Lane g, 1-kilobase ladder. Southern blots were probed with ^{32}P-labeled pS86b.

and f of Fig. 5). Therefore, the number of fragments containing pJEL101-related sequences was considerably lower when compared to strains of *X. c.* pv. *oryzae* from other areas. A bacterium identified as a *Xanthomonas* spp., which was isolated from rice but was not pathogenic to it, did not hybridize with pJEL101 (Fig. 5, lane d).

pJEL101 also hybridized with DNA digests from eight of 25 pathovars of *X. campestris*. Of the strains with DNA that hybridized, only *X. c.* pv. *oryzicola* contained a quantity of bands similar to that observed in *X. c.* pv. *oryzae* (Fig. 6, lanes m and n). The number of bands was estimated to be less than 25 from visual inspection of the filter, and thus, the actual number of copies in each strain was likely to be fewer than the copies found in strains of *X. c.* pv. *oryzae* (81), with the exception of the strains from the United States. DNA from *X. c.* pv. *holcicola* (Fig. 6, lanes c and d), *X. c.* pv. *vasculorum* (Fig. 6, lanes i and j), *X. c.* pv. *phleipratensis* (Fig. 6, lanes k and l), and *X. c.* pv. *pelargonii* (not shown) also hybridized to pJEL101 in a manner indicative of a repetitive sequence, although the number of bands in each case was below 10. One or two bands also were detected in DNA from some other pathovars (*X. c.* pv. *vesicatoria*, lanes o and p of Fig. 6; *X. c.* pv. *secalis* and *X. c.* pv. *undulosa*, not shown).

Hybridization of pJEL101 to DNA from seven additional strains of *X. c.* pv. *oryzicola* resulted in patterns as complex as those previously observed with strains BLS175 and BLS256 (Fig. 7). The RFLP patterns of the nine strains appeared different from the patterns for strains of *X. c.* pv. *oryzae*. The proportion of mismatched fragments calculated for all pairwise combinations of strains of *X. c.* pv. *oryzae* vs *X. c.* pv. *oryzicola* ranged from 0.84–0.93 as compared to 0.08–0.36 for comparisons of strains of *X. c.* pv. *oryzae* on the same blot. The proportion of mismatched fragments calculated for all pairwise combinations of the nine strains of *X. c.* pv. *oryzicola* ranged from 0.08–0.69, whereas combinations of *X. c.* pv. *oryzae* from different geographic areas (Fig. 5, excluding those from the United States) ranged from 0.03–0.57. Thus,

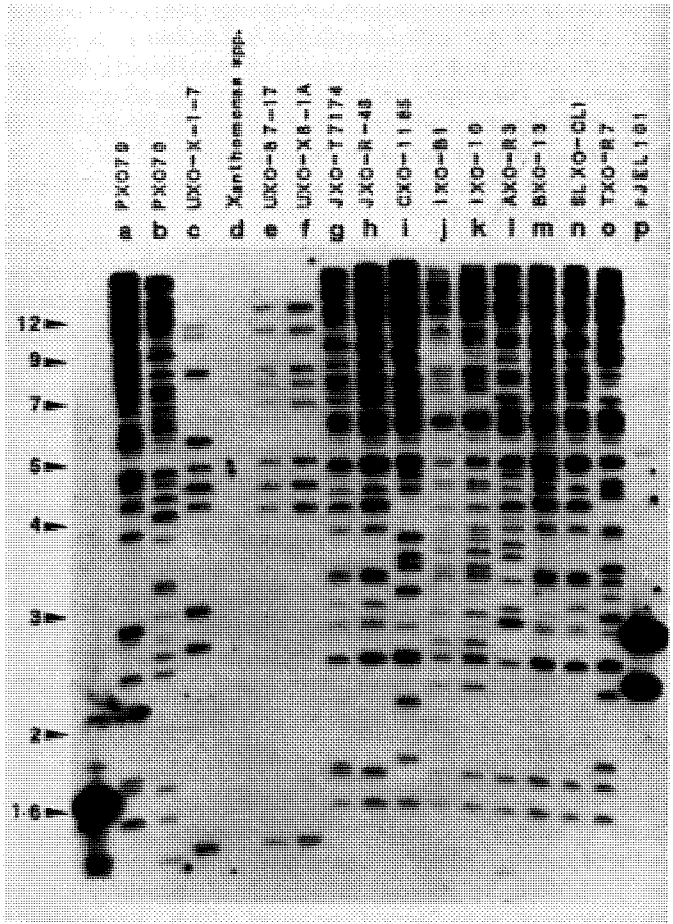

Fig. 5. Southern blot analysis of total DNA from strains of *Xanthomonas campestris* pv. *oryzae* representing different geographic areas. The probe was ^{32}P-labeled pJEL101. Lanes a and b, PXO79 and PXO70 (the Philippines); lane c, UXO-X-1-7 (Texas, United States); lane d, *Xanthomonas* spp. (Texas, United States); lanes e and f, UXO-87-17 and UXO-X8-1A (Louisiana, United States); lanes g and h, JXO-T7174 and JXO-R-48 (Japan); lane i, CXO-1185 (Colombia); lane j, IXO-B1 (Bangladesh); lane k, IXO-10 (India); lane l, AXO-R3 (Australia); lane m, BXO-13 (Burma); lane n, SLXO-CL1 (Sri Lanka); and lane o, TXO-R7 (Thailand). Lane p, *Eco*RI-*Hin*dIII-digested pJEL101. Genomic DNA was digested with *Eco*RI.

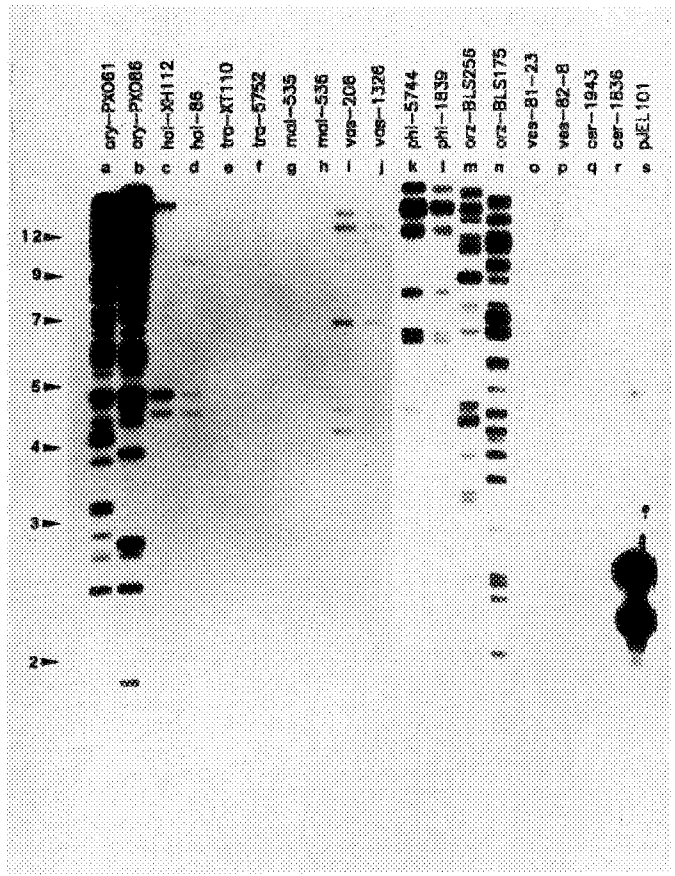

Fig. 6. Southern blot analysis of total DNA from *Xanthomonas campestris* pathovars. The probe was ^{32}P-labeled pJEL101. *X. c.* pv. *oryzae* PXO61 (lane a) and PXO86^{Rif} (lane b); *X. c.* pv. *holcicola* XH112 (lane c) and 86 (lane d); *X. c.* pv. *translucens* XT110 (lane e) and 5752 (lane f); *X. c.* pv. *malvacearum* 535 (lane g) and 536 (lane h); *X. c.* pv. *vasculorum* 206 (lane i) and 1326 (lane j); *X. c.* pv. *phleipratensis* 5744 (lane k) and 1839 (lane l); *X. c.* pv. *oryzicola* BLS256 (lane m) and BLS175 (lane n); *X. c.* pv. *vesicatoria* 81-23 (lane o) and 82-8 (lane p); and *X. c.* pv. *cerealis* 1943 (lane q) and 1836 (lane r). Lane s, *Eco*RI-*Hin*dIII-digested pJEL101. Genomic DNA was digested with *Eco*RI.

although the repetitive element was fairly polymorphic within *Eco*RI-digested DNA from each pathovar, the two pathovars could clearly and easily be distinguished by hybridization patterns with pJEL101.

DISCUSSION

Hybridization between total genomic DNA and individual genomic clones revealed the presence of two highly repetitive sequences in *X. c.* pv. *oryzae*. The two elements, contained in pJEL101 and pS86d, are distinct elements because genomic sequences of the two clones did not cross-hybridize and, in addition, hybridization patterns with genomic DNA digests were different. Repetitive sequences of pJEL101 were detected in all strains of *X. c.* pv. *oryzae* and some other pathovars of *X. campestris*.

The discovery of repetitive elements is not unique to *X. c.* pv. *oryzae*. More noteworthy is the prevalence of repetitive sequences in the genome of *X. c.* pv. *oryzae*.

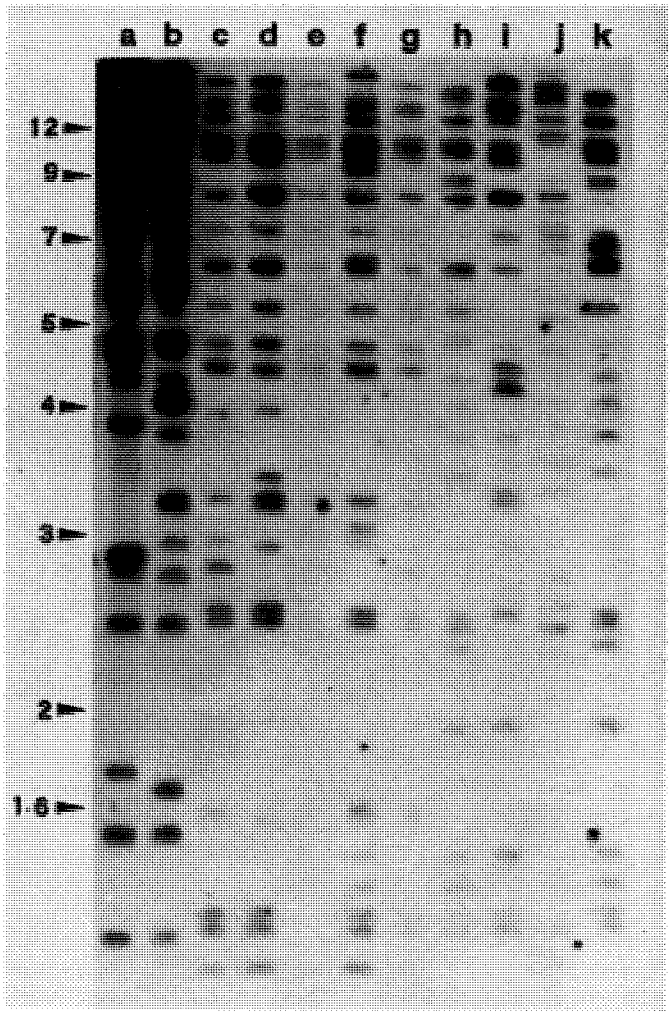

Fig. 7. Southern blot analysis of genomic DNA from *Xanthomonas campestris* pv. *oryzicola*. The probe was ^{32}P-labeled pJEL101. Strains PXO86^{Rif} and PXO61 of *X. c.* pv. *oryzae* (lanes a and b, respectively). Strains of *X. c.* pv. *oryzicola*: BLS303 (lane c), BLS298 (lane d), BLS295 (lane e), BLS292 (lane f), BLS290 (lane g), BLS288 (lane h), BLS256 (lane i), BLS179 (lane j), and BLS175 (lane k). Genomic DNA was digested with *Eco*RI.

For example, pJEL101 hybridized to 85% of the pSa747 clones of PXO86^{Rif} tested, indicating that the element is distributed throughout the genome. Clone pS86d contains a different repetitive sequence, indicating that repetitive sequences are ubiquitous components of the genome of *X. c.* pv. *oryzae*.

The repetitive element occupies about 1 kb of the *Eco*RI-*Hin*dIII fragment in pJEL101, based on hybridization analysis of deleted mutants. However, a more detailed analysis is necessary to determine if the whole repetitive element is contained within pJEL101. Since there is no clear indication of the borders of repetititve elements, sequence analysis of several different clones containing related elements will be required.

Quantitation of the slot blot hybridization provided an estimate of 81 copies of the element in pJEL101 per genome. The copy number (≈ 81) and size (≈ 1.0 kb) of the pJEL101 element are most similar to the copy number (2–40) and size (0.7–10 kb) of insertion sequences (Appelbaum *et al.* 1985; Comai and Kosuge 1983; Flores *et al.* 1987; Iida *et al.* 1983; Kaluza *et al.* 1985; Kleckner 1981; McLafferty *et al.* 1988; Mogen and Oleson 1987; Ruvkun *et al.* 1982), although there is no evidence that the pJEL101 element transposes. The copy number of the repetitive sequence(s) in pS86d appears to be similar to that of the element in pJEL101, based on Southern hybridizations.

All strains of *X. c.* pv. *oryzae* tested from various geographic areas hybridized extensively with pJEL101. The fact that all strains of *X. c.* pv. *oryzae* contain the repetitive element in high copy indicates the evolution of the pathovar as a distinct clonal population. The exceptional strains were all from recent collections from the United States (Jones *et al.* 1989). Fatty acid profiles, physiological and biochemical tests, restriction endonuclease analysis, serological identification, host range, and symptoms on rice grouped the strains from the United States more closely to *X. c.* pv. *oryzae* than to other pathovars of *X. campestris*. The strains from the United States, however, are not as aggressive to rice as strains from Asia. Strains from Asia and the United States also can be differentiated by monoclonal antibodies generated to strains from the United States. Hybridization with pJEL101 provides additional evidence of differences between the strains at the genomic level.

The 125 pathovars of *X. campestris* are differentiated from one another on the basis of host range and symptoms on a particular host (Bradbury 1984; Leyns *et al.* 1984). For example, *X. c.* pv. *oryzae* is differentiated from other pathovars of *X. campestris* by its specificity to rice and from *X. c.* pv. *oryzicola* by the difference in symptoms induced on rice (blight vs streak). *X. c.* pv. *oryzae* cannot be identified solely on the basis of biochemical or physiological tests (Vera Cruz *et al.* 1984). Examination of pathovars of *X. campestris* showed that the pJEL101 sequence could be used as a probe for identification of *X. c.* pv. *oryzae* by RFLP analysis. Only one other species (*X. c.* pv. *oryzicola*) possessed a complex hybridization pattern, and the pattern was distinct from that of all strains of *X. c.* pv. *oryzae*. The high complexity of the signal for *X. c.* pv. *oryzicola* was intriguing because it is also a rice pathogen. The presence in high copy of the pJEL101 element in all the strains of *X. c.* pv. *oryzicola* prompts

speculation that the pathovar is evolutionarily more closely related to *X. c.* pv. *oryzae* than to other pathovars. The differences in hybridization patterns and copy numbers of the element between the *X. c.* pv. *oryzae* and *X. c.* pv. *oryzicola*, however, suggest a clear divergence of the two populations.

The pJEL101 clone could possibly serve for diagnosis of strains from other pathovars of *X. campestris*. Nine strains of *X. c.* pv. *oryzicola* were found to have characteristic band patterns. Strains of *X. c.* pv. *holcicola*, *X. c.* pv. *vasculorum*, and *X. c.* pv. *phleipratensis* showed similar or identical patterns within the same pathovar designation; no two pathovars had the same pattern. Although pathovar-specific patterns were present, more strains of each pathovar from various geographic locations need to be examined. There was no correlation between type of host plant (monocot vs dicot) and hybridization with pJEL101. It is not known if strains of other pathovars whose DNA hybridized with pJEL101, other than *X. c.* pv. *oryzicola*, are pathogenic to rice. Additional sequences of pJEL101 that are not included in the 1-kb repetitive sequence of *X. c.* pv. *oryzae* may have hybridized with the DNA from other *X. campestris* pathovars.

Repetitive sequences provide an alternative strategy for RFLP analysis of genetic relationships among strains of *X. c.* pv. *oryzae*. Their dispersed nature allows examination of many segments of the genome at one time. Repetitive sequences may also provide sensitive probes for pathovar diagnosis due to the high copy number. However, the presence of related sequences in other pathovars of *X. campestris* precludes the use of pJEL101 in dot hybridization procedures (Gilbertson *et al.* 1989). Other repetitive sequences and specific sequences in the pJEL101 element are currently being examined for pathovar specificity.

Further characterization of the repetitive sequences is required to determine what type or class of repetitive DNA is present in pJEL101. However, the high copy number of the pJEL101 element, the ubiquity of the sequence, the variability of hybridization patterns among strains of the same pathovar, and the presence of a similar element in a related rice pathogen (*X. c.* pv. *oryzicola*) compel speculation that the pJEL101 sequence, as well as others, may be involved in genetic adaptation of strains to diverse rice cultivars.

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