Characterization of Strains of *Xanthomonas campestris* pv. *holcicola* by PAGE of Membrane Proteins and by REA and RFLP Analysis of Genomic DNA

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ABSTRACT

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Polyacrylamide gel electrophoresis (PAGE) of membrane proteins, restriction endonuclease analysis (REA), and restriction fragment length polymorphism (RFLP) analysis of genomic DNA were used to characterize strains of *Xanthomonas campestris* pv. *holcicola*. After PAGE, a common membrane protein pattern was observed for 19 strains of *X. c.* pv. *holcicola* from Australia, Lesotho, Mexico, New Zealand, the Republic of South Africa, and the United States of America. This profile was distinct from the 22 other pathovars of *X. campestris* tested. Differences in fragmentation patterns were observed between strains of *X. c.* pv. *holcicola* after electrophoretic separation of EcoRI-digested genomic DNA in agarose gels (REA) or hybridization analysis after blotting the separated DNA to membranes (RFLP). However, DNA fragmentation patterns were distinguishable by both REA and RFLP, which were common to all strains of *X. c.* pv. *holcicola* but different from strains of other *Xanthomonas* pathovars tested.

Bacterial diseases of sorghum (*Sorghum bicolor* (L.) Moench) have been reported in all major sorghum-growing areas of the world. Bacterial leaf stripe caused by *Pseudomonas andropogonis* (Smith) Stapp, bacterial leaf spot caused by *P. syringae* pv. *syringae* (van Hall), and bacterial leaf streak caused by *Xanthomonas campestris* pv. *holcicola* (Elliott) Dye, are the most prevalent diseases. Bacterial leaf stripe is the most common of the three in most parts of the world except the Republic of South Africa (D. Nowell, Pioneer Seed Company, RSA [PTY] Limited, Greytown, Natal, South Africa, *personal communication*) and Lesotho (Qhobela and Claflin, *unpublished*), where it has not been reported. In these two countries, bacterial leaf streak is the only bacterial disease found in sorghum. *X. c.* pv. *holcicola* is currently characterized by host range studies (1). Our objective was to find techniques that could be used to characterize *X. c.* pv. *holcicola*, and which might later be used to diagnose bacterial leaf streak of sorghum.

Characterization of organisms by polyacrylamide gel electrophoresis (PAGE) of cellular proteins is based on the identification of specific protein patterns (6,15,17). The protein banding patterns are compared visually for simplicity of identification, although numerical

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Table 1. Geographical distribution of *Xanthomonas campestris* pv. *holcicola* strains used

Strain number[a]	Host isolated from	Locality
PDDCC 3103[b]	*Sorghum bicolor*	New Zealand
NCPPB 1241	*Sorghum bicolor*	Australia
ATCC 13461	*Holcus* spp.	Texas, USA
TX-1	*Sorghum bicolor*	Texas, USA
KS 66	*Sorghum bicolor*	Kansas, USA
KS 86	*Sorghum bicolor*	Kansas, USA
KS 93	*Sorghum bicolor*	Kansas, USA
MEX 1A	*Sorghum bicolor*	Mexico
LES 107	*Sorghum bicolor*	Lesotho
LES 114	*Sorghum bicolor*	Lesotho
LES 116	*Sorghum bicolor*	Lesotho
LES 123	*Sorghum bicolor*	Lesotho
LES 124	*Sorghum bicolor*	Lesotho
SAS 211	*Sorghum bicolor*	South Africa
SAS 212	*Sorghum bicolor*	South Africa
SAS 213	*Sorghum bicolor*	South Africa
SAS 214	*Sorghum bicolor*	South Africa
SAS 215	*Sorghum bicolor*	South Africa
SAS 216	*Sorghum bicolor*	South Africa

[a]Strains were obtained from the American Type Culture Collection, Rockville, MD (ATCC), The National Collection of Plant Pathogenic Bacteria, Hertfordshire, United Kingdom (NCPPB), Plant Disease Division Culture Collection, Auckland, New Zealand (PDDCC), and Dr. L. E. Claflin, Department of Plant Pathology, Kansas State University, Manhattan, KS (all other strain designations).

[b] Pathotype strain.

analysis methods have been developed (2,14,16). In addition to this technique, we also used restriction endonuclease analysis (REA) and restriction fragment length polymorphism (RFLP) analysis of the genomic DNA to achieve the same goals. The latter two techniques have also been used to classify and differentiate other pathovars of *X. campestris* (4,5,7). The REA procedure is based on the identification of specific fragment patterns after cleavage of DNA with a restriction enzyme (9). The number and location of endonuclease restriction sites along the DNA strand are unique for each genome, thus each genome will have a unique fragmentation pattern. The REA procedure is useful for side-by-side comparison of samples run on the same gel, but comparisons between different gels is difficult. In addition, the patterns are often complex and difficult to interpret. The RFLP procedure, although dependent on the ability of the chosen probe to identify unique polymorphisms, simplifies the analysis of DNA fragmentation patterns and usually obviates the need for side-by-side comparisons in a single gel.

MATERIALS AND METHODS

Bacterial cultures. All strains of *X. c.* pv. *holcicola* were determined to be pathogenic on sorghum (Cultivar 80B3039, D. T. Rosenow, Texas Agricultural Experiment Station, Lubbock, TX) by a previously described method (13). All bacterial strains were maintained at 28 C on yeast-extract, dextrose, calcium carbonate agar medium (12), and lyophilized for long-term storage. Sources of bacterial strains and geographical distribution are listed in Tables 1 and 2.

PAGE of membrane proteins. Cell membrane proteins of all bacterial strains were isolated according to the method of Qhobela and Claflin (12). Approximately 10–20 μg of total protein was electrophoresed as previously described (12) in a vertical 12.5% polyacrylamide gel (17 cm × 15 cm). The gels were stained with Coomassie brilliant blue (0.125% [w/v] Coomassie brilliant blue R, 50% methanol, and 10% glacial acetic acid) for 12 hr and destained (50% methanol, 10% glacial acetic acid) until the background was clear. The resultant patterns from the 19 strains of *X. c.* pv. *holcicola* were visually compared to membrane protein patterns of strains from 22 other pathovars of *X. campestris* and two *Pseudomonas* spp. (Table 2). At least two strains each of all the organisms listed in Table 2 were used in the study, except that only one strain was available for *X. campestris* pv. *carotae* (Kendrick) Dye and *X. campestris* pv. *pisi* (Goto & Okabe) Dye.

REA and RFLP analysis of genomic DNA. Total DNA was extracted from bacterial strains by a modification of the method of Owen and Borman (11) as described by Leach et al (8). The DNA (10 μg) was digested to completion with three restriction enzymes (EcoR1, BamH1, and BstEII) at 2 U/ μg DNA as suggested by the manufacturer (Bethesda Research Laboratories [BRL] Bethesda, MD). The DNA fragments (2 μg/well) were separated by electrophoresis (2.5 volts per cm for 12 hr) in a horizontal 0.7% agarose gel (20 cm × 21.5 cm) immersed in Tris-borate buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM Na_2EDTA, pH 8.0). A 1-kilobase ladder (BRL) was included as a size standard. The gels were immersed in a solution of ethidium bromide (0.5 μg/ml) to stain the DNA fragments and examined under ultraviolet irradiation.

For REA of genomic DNA, *X. c.* pv. *holcicola* was characterized by visual assessment of unique fragment bands within the total genomic profile. For RFLP analysis of genomic DNA, the digested DNA (5 μg/well) was transferred onto nylon membranes (GeneScreen Plus, NEF-976, New England Nuclear Research Products, Boston, MA); Southern hybridizations and high stringency washes were done as recommended by the membrane manufacturer. The membranes were probed with pJEL101, a pUC18 derivative containing a 2.4 Kb repetitive DNA element from *X. c.* pv. *oryzae* (8). The reason for using pJEL101 was its initial promise as a probe for characterizing pathovars of *X.*

Table 2. Strains of other *Xanthomonas campestris* pathovars and two *Pseudomonas* pathogens of sorghum used and information on their origin and isolation

Organism	Strain[a]	Host isolated from	Locality
X. campestris pv. *alfalfae*	KX-1	*Medicago sativa*	Kansas
	Alf-702	*Medicago sativa*	Kansas
X. campestris pv. *begoniae*	NCPPB 1926[b]	*Begonia* spp.	New Zealand
	KSU 4515	*Begonia* spp.	Kansas
X. campestris pv. *campestris*	NCPPB 528[b]	*Brassica* spp.	New Zealand
	KSU 4518	*Brassica* spp.	Kansas
X. campestris pv. *carotae*	NCPPB 1422[b]	*Daucus carota*	Hungary
X. campestris pv. *cerealis*	NCPPB 1836	*Secale cereale*	USA
	NCPPB 1943	*Hordeum vulgare*	USA
X. campestris pv. *cucurbitae*	NCPPB 2597[b]	*Cucumis sativa*	New Zealand
	KSU 4461	*Cucumis sativa*	Kansas
X. campestris pv. *glycines*	PDDCC 2336	*Glycine max*	New Zealand
	KSU 1432	*Glycine max*	Kansas
X. campestris pv. *graminis*	NCPPB 2695	*Lolium multiflorum*	Switzerland
	NCPPB 2700[b]	*Dactylis glomerata*	Switzerland
X. campestris pv. *hedereae*	PDDCC 1661	*Hedera helix*	New Zealand
	KSU 4497	*Hedera helix*	Nebraska
X. campestris pv. *malvacearum*	KSU 4485	*Gossypium hirsutum*	Texas
	KSU 28	*Gossypium hirsutum*	Texas
X. campestris pv. *oryzae*	PXO 124	*Oryza sativa*	Philippines
	PXO 107	*Oryza sativa*	Philippines
X. campestris pv. *oryzicola*	PDDCC 5743[b]	*Oryza sativa*	Malaysia
	BLS 288	*Oryza sativa*	Philippines
X. campestris pv. *phaseoli*	NCPPB 2064	*Lablab purpureus*	Sudan
	NCPPB 1138	*Phaseolus vulgaris*	Zambia
X. campestris pv. *phleipratensis*	NCPPB 5749	*Phleum pratense*	USA
	NCPPB 5744[b]	*Phleum pratense*	USA
X. campestris pv. *pennamericanum*	N'D 116	*Pennisetum glaucum*	Niger
	N'D 150	*Pennisetum glaucum*	Niger
X. campestris pv. *pisi*	NCPPB 762[b]	*Pisum sativum*	Japan
X. campestris pv. *secalis*	PDDCC 5749	*Secale cereale*	Canada
	XT-104	*Secale cereale*	Georgia
X. campestris pv. *translucens*	PDDCC 5752[b]	*Hordeum vulgare*	USA
	XT-116	*Triticum aestivum*	USA
X. campestris pv. *undulosa*	NCPPB 1945	*Triticum aestivum*	Canada
	PDDCC 5755	*Triticum aestivum*	Canada
X. campestris pv. *vasculorum*	PDDCC 5757[b]	*Saccharum officinarum*	Mauritius
	MA 2771	*Saccharum officinarum*	Mauritius
X. campestris pv. *vesicatoria*	KSU 75-3	*Lycopersicon* spp.	Florida
	KSU 82-7	*Capsicum* spp.	Florida
X. campestris pv. *vignicola*	KSU 4659	*Vigna unguiculata*	Niger
	CP-2	*Vigna unguiculata*	Niger
Pseudomonas andropogonis	KS 74	*Sorghum bicolor*	USA
	KS 945	*Sorghum bicolor*	Australia
P. syringae pv. *syringae*	PDDCC 4415	*Sorghum bicolor*	Yugoslavia
	PDDCC 4474	*Sorghum bicolor*	Australia

[a] Strains were obtained from: Plant Disease Division Culture Collection, Auckland, New Zealand (PDDCC), National Collection of Plant Pathogenic Bacteria, Hertfordshire, United Kingdom (NCPPB), and Dr. L. E. Claflin, Department of Plant Pathology, Kansas State University, Manhattan, KS (all other strain designations).

[b] Pathotype strain.

campestris (8). The clone was labeled with a [α-^{32}P]-dCTP using a nick translation kit (BRL). The blots were prehybridized at 65 C for 12 hr in a solution of 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer (PB, pH 7.0), 1.0 M NaCl, and 300 μg/ml denatured herring testes DNA. For hybridization, denatured labeled probe DNA (10^6 cpm/ml) was added directly to the prehybridization solution, and the blots were incubated at 65 C for 18 hr. To facilitate estimation of fragment sizes, [α-^{32}P]-dCTP end-labeled 1-kilobase ladder was added to the hybridization solution. After hybridization, the blots were washed twice at 65 C in 2× salt sodium citrate (SSC) (1× SSC is 3 M NaCl and 0.3 M $Na_3C_6H_5O_7$) containing 0.1% SDS and 5 mM PB, then twice in 0.5× SSC containing 0.1% SDS and 3 mM PB. Autoradiographic exposures were conducted for 24–48 hr at −80 C using Cronex film (Du Pont) and a Du Pont Cronex Hi-Plus intensifying screen.

RESULTS AND DISCUSSION

PAGE of membrane proteins. A common membrane protein pattern was observed for all strains of *X. c.* pv. *holcicola* in a 12.5% SDS polyacrylamide gel (Fig. 1). The overall protein profile observed for strains of *X. c.* pv. *holcicola* was different than the patterns from other pathovars of *X. campestris* (Figs. 2 and 3). In addition to the overall protein profile, three proteins (approximately 49,000, 32,000, and 21,000 daltons) selected in the protein profile of *X. c.* pv. *holcicola* (Fig. 1) could be used to differentiate *X. c.* pv. *holcicola* from other pathovars of *X. campestris*

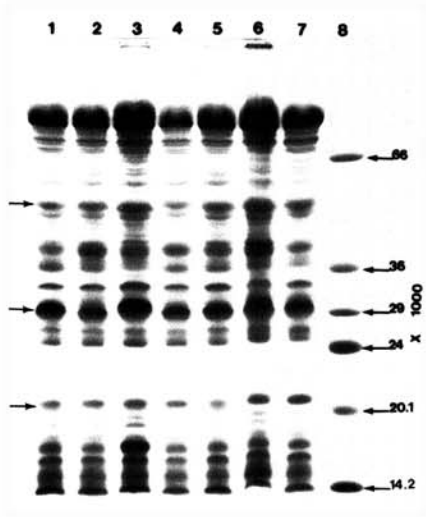

Fig. 1. Membrane protein profiles of *Xanthomonas campestris* pv. *holcicola*. Strains PDDCC 3103 (Lane 1), NCPPB 1241 (Lane 2), KS 66 (Lane 3), LES 107 (Lane 4), LES 124 (Lane 5), SAS 211 (Lane 6), SAS 213 (Lane 7), and SDS-7 Dalton mark VII-L molecular weight marker (Lane 8). The three arrows illustrate the 21,000, 32,000, and 49,000 dalton proteins observed in *X. c.* pv. *holcicola*.

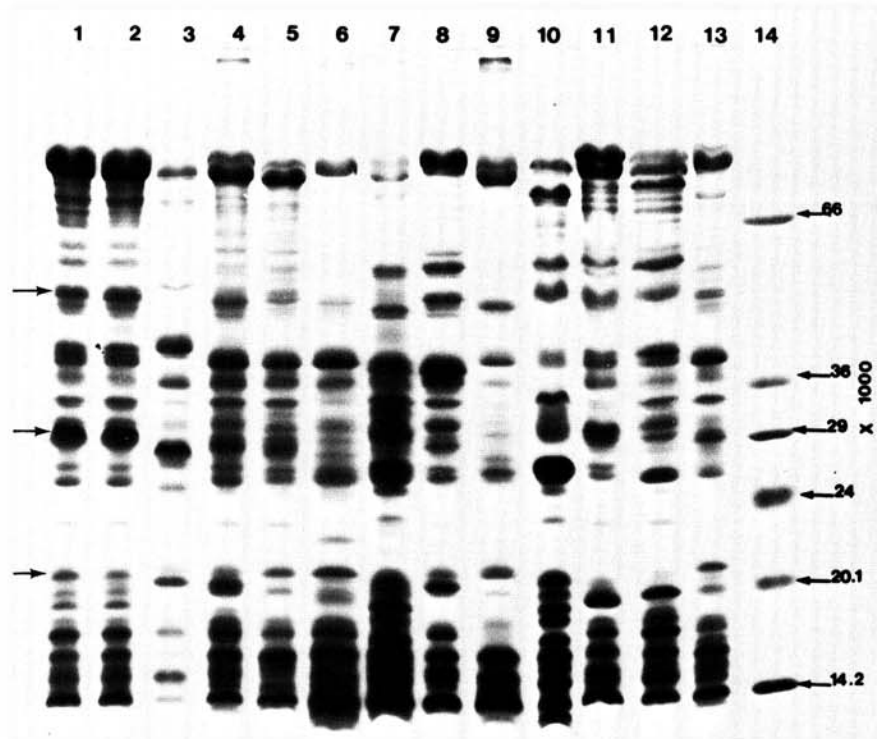

Fig. 2. Membrane protein profiles of *Xanthomonas campestris* pathovars. *X. c.* pv. *holcicola* (Lanes 1 and 2), *X. c.* pv. *vasculorum* (Lane 3), *X. c.* pv. *alfalfae* (Lane 4), *X. c.* pv. *begoniae* (Lane 5), *X. c.* pv. *campestris* (Lane 6), *X. c.* pv. *cerealis* (Lane 7), *X. c.* pv. *cucurbitae* (Lane 8), *X. c.* pv. *glycines* (Lane 9), *X. c.* pv. *graminis* (Lane 10), *X. c.* pv. *oryzicola* (Lane 11), *X. c.* pv. *oryzae* (Lane 12), *X. c.* pv. *hedereae* (Lane 13), and SDS-7 Dalton mark VII-L molecular weight marker (Lane 14). The three arrows illustrate the 21,000, 32,000, and 49,000 dalton proteins observed in *X. c.* pv. *holcicola*.

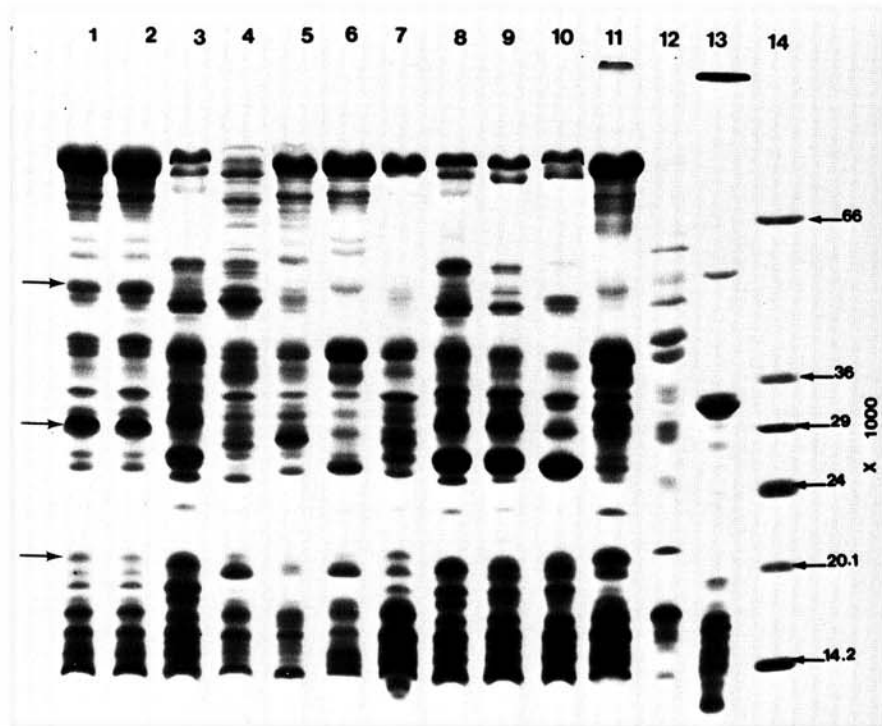

Fig. 3. Membrane protein profiles of selected *Xanthomonas campestris* pathovars and two *Pseudomonas* spp. infecting sorghum. *X. c.* pv. *holcicola* (Lanes 1 and 2), *X. c.* pv. *secalis* (Lane 3), *X. c.* pv. *phaseoli* (Lane 4), *X. c.* pv. *malvacearum* (Lane 5), *X. c.* pv. *pennamericanum* (Lane 6), *X. c.* pv. *vignicola* (Lane 7), *X. c.* pv. *translucens* (Lane 8), *X. c.* pv. *undulosa* (Lane 9), *X. c.* pv. *phleipratensis* (Lane 10), *X. c.* pv. *vesicatoria* (Lane 11), *P. andropogonis* (Lane 12), *P. avenae* (Lane 13), and SDS-7 Dalton mark VII-L molecular weight marker (Lane 14). The three arrows illustrate the 21,000, 32,000, and 49,000 dalton proteins observed in *X. c.* pv. *holcicola*.

examined. Although the 49,000 and 32,000 dalton proteins were present in the profile of *X. c.* pv. *oryzicola* (Fig. 2, lane 11), the 21,000 dalton protein was not. Protein profiles from two other bacterial pathogens of sorghum, *P. andropogonis* and *P. syringae* pv. *syringae*, were also distinctive from strains of *X. c.* pv. *holcicola* (Fig. 3).

REA and RFLP of genomic DNA. Only fragmentation patterns generated after digestion from EcoR1 are presented, as they were easier to interpret than those generated by digestion with BamH1 or BstEII. Differences were present between strains. For example, four strains of *X. c.* pv. *holcicola* (PDDCC 3103, KS 93, TX-1, and MEX 1A) each had an intense band at approximately 0.8 Kb (Fig. 4, lanes 1, 3, 5, and 7) that was possibly plasmid DNA. However, a DNA fragmentation pattern common to all strains of *X. c.* pv. *holcicola* was observed (Fig. 4). From the pattern, three easily observed features were selected (a broad space at approximately 5.0 Kb and a pair of bands at approximately 3.7 and 3.9 Kb) that, as a group, were common to all strains of *X. c.* pv. *holcicola* (Fig. 4), but not to other pathovars of *X. campestris* examined (Fig. 5).

RFLP analysis of DNA from *X. c.* pv. *holcicola* revealed two distinct bands, one greater than 12 Kb and another at 4.8 Kb (Fig. 6). DNA from four strains (PDDCC 3103, KS 93, KS 86, and ATCC 13461) exhibited an additional band at 8 Kb (Fig. 6, lanes 1, 3, 4, and 6). Significance of the presence or absence of the 8-Kb band is currently unknown. The pattern observed after hybridization of EcoRl-digested DNA from *X. c.* pv. *holcicola* with pJEL101 was distinctly different from the patterns of other pathovars of *X. campestris* (Fig. 7). Therefore, pJEL101 may be useful as a probe to differentiate *X. c.* pv. *holcicola* from other pathovars of *X. campestris*.

Our objectives were to use PAGE of membrane proteins and REA and RFLP analysis of genomic DNA to characterize strains of *X. c.* pv. *holcicola* from various geographical areas. Representative patterns were identified after the three techniques that were characteristic to our strains of *X. c.* pv. *holcicola* and yet were distinct from strains of all other pathovars of *X. campestris* tested. The total protein profiles observed after PAGE were strikingly similar for all strains of *X. c.* pv. *holcicola*. Differences between strains of *X. c.* pv. *holcicola* were more easily detected using REA and RFLP techniques than using PAGE analysis. Thus, with all three techniques, patterns characteristic of the pathovar can be identified. However, obvious differences between strains were only apparent after REA and RFLP analyses.

Of the three techniques used, PAGE of membrane proteins was simpler to

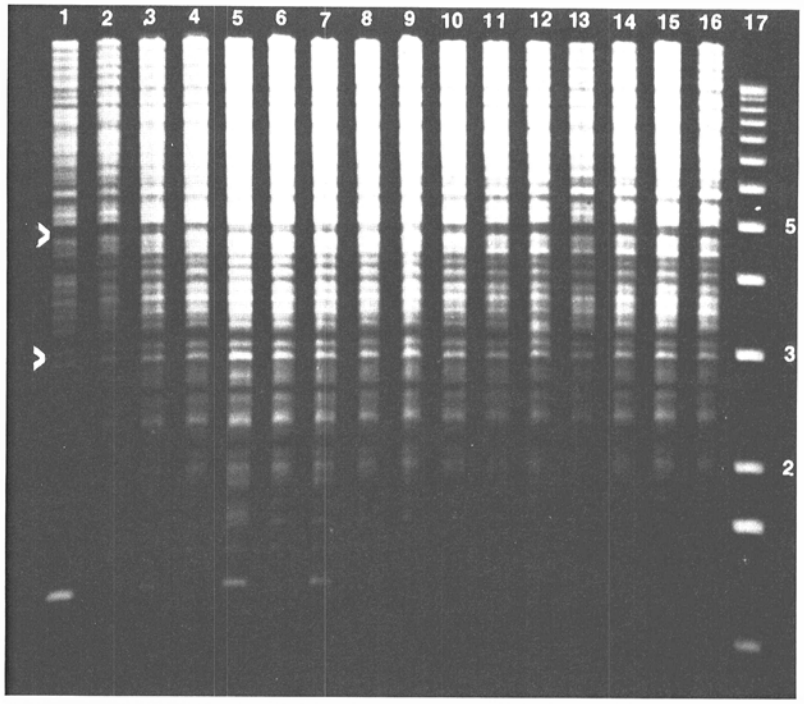

Fig. 4. EcoR1 digested genomic DNA from *Xanthomonas campestris* pv. *holcicola*. Strains PDDCC 3103 (Lane 1), NCPPB 1241 (Lane 2), KS 93 (Lane 3), KS 86 (Lane 4), TX-1 (Lane 5), ATCC 13461 (Lane 6), MEX 1A (Lane 7), LES 114 (Lane 8), LES 123 (Lane 9), LES 124 (Lane 10), SAS 211 (Lane 11), SAS 212 (Lane 12), SAS 213 (Lane 13), SAS 214 (Lane 14), SAS 215 (Lane 15), SAS 216 (Lane 16), and 1 Kb ladder marker (Lane 17). The two arrows illustrate the broad space and pair of bands that were unique to *X. c.* pv. *holcicola*.

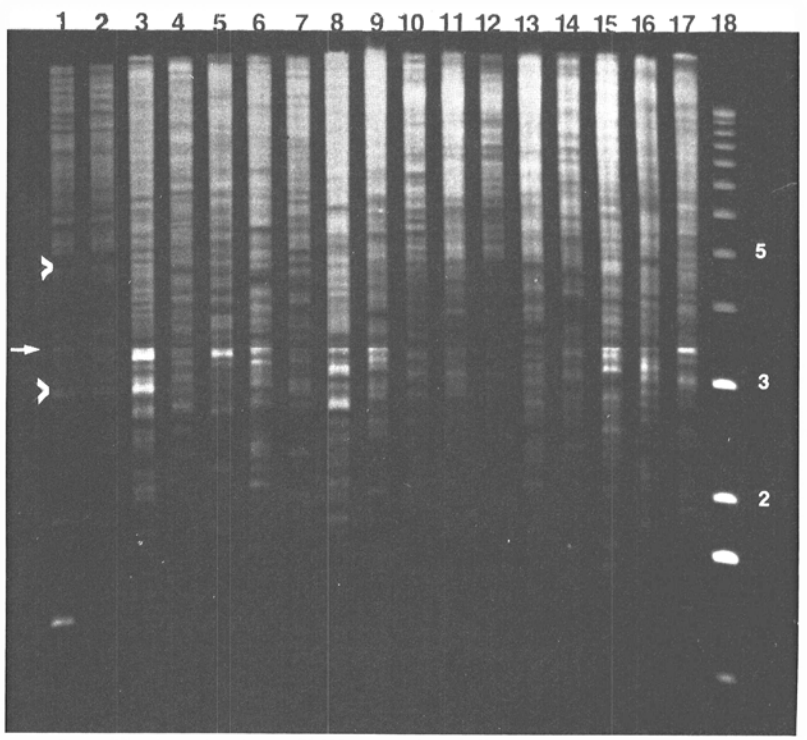

Fig. 5. EcoR1 digested genomic DNA from selected *Xanthomonas campestris* pathovars. *X. c.* pv. *holcicola* (Lanes 1 and 2), *X. c.* pv. *alfalfae* (Lane 3), *X. c.* pv. *begoniae* (Lane 4), *X. c.* pv. *campestris* (Lane 5), *X. c.* pv. *cucurbitae* (Lane 6), *X. c.* pv. *glycines* (Lane 7), *X. c.* pv. *phaseoli* (Lane 8), *X. c.* pv. *pisi* (Lane 9) *X. c.* pv. *translucens* (Lane 10), *X. c.* pv. *undulosa* (Lane 11), *X. c.* pv. *secalis* (Lane 12), *X. c.* pv. *graminis* (Lane 13), *X. c.* pv. *phleipratensis* (Lane 14), *X. c.* pv. *oryzae* (Lane 15), *X. c.* pv. *vasculorum* (Lane 16), *X. c.* pv. *vesicatoria* (Lane 17), and 1 Kb ladder marker (Lane 18). The two arrows illustrate the broad space and pair of bands that were unique to *X. c.* pv. *holcicola*.

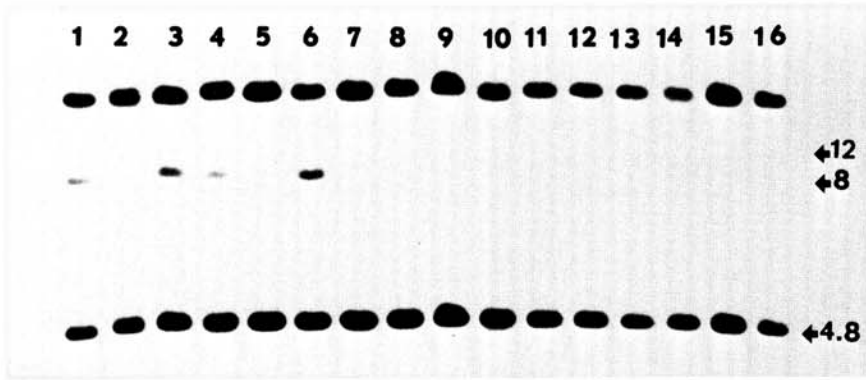

Fig. 6. Southern blot analysis of EcoR1 digested genomic DNA from *Xanthomonas campestris* pv. *holcicola* strains probed with ^{32}P-labeled pJEL101. Strains PDDCC 3103 (Lane 1), NCPPB 1241 (Lane 2), KS 93 (Lane 3), KS 86 (Lane 4), TX-1 (Lane 5), ATCC 13461 (Lane 6), Mex 1A (Lane 7), LES 114 (Lane 8), LES 123 (Lane 9), LES 124 (Lane 10), SAS 211 (Lane 11), SAS 212 (Lane 12), SAS 213 (Lane 13), SAS 214 (Lane 14), SAS 215 (Lane 15), and SAS 216 (Lane 16).

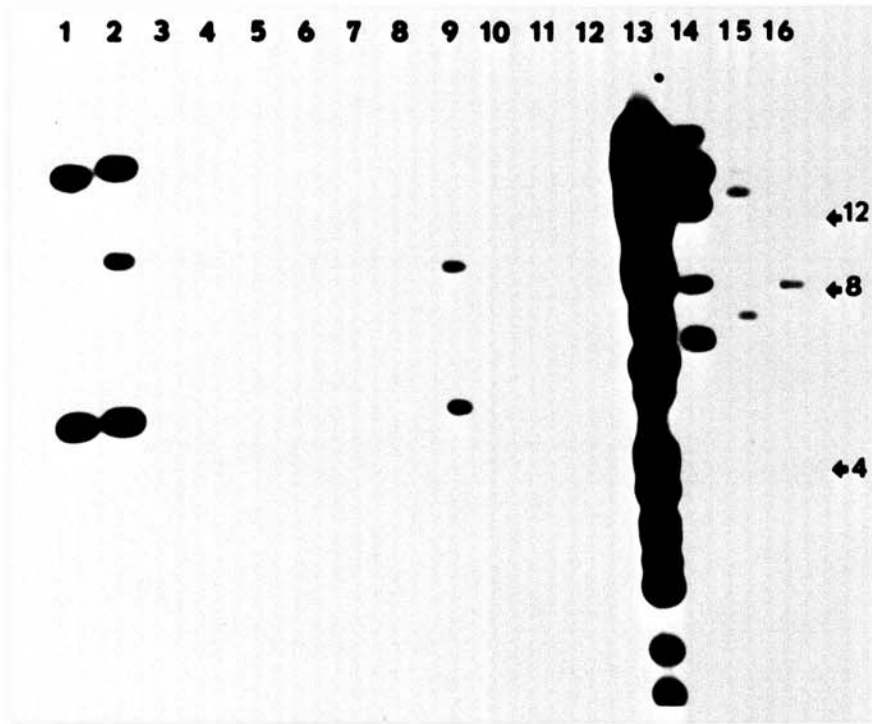

Fig. 7. Southern blot analysis of EcoR1 digested genomic DNA from selected *Xanthomonas campestris* pathovars probed with ^{32}P-labeled pJEL101. *X. c.* pv. *holcicola* (Lanes 1 and 2), *X. c.* pv. *alfalfae* (Lane 3), *X. c.* pv. *begoniae* (Lane 4), *X. c.* pv. *campestris* (Lane 5), *X. c.* pv. *cucurbitae* (Lane 6), *X. c.* pv. *glycines* (Lane 7), *X. c.* pv. *phaseoli* (Lane 8), *X. c.* pv. *pisi* (Lane 9) *X. c.* pv. *translucens* (Lane 10), *X. c.* pv. *undulosa* (Lane 11), *X. c.* pv. *secalis* (Lane 12), *X. c.* pv. *graminis* (Lane 13), *X. c.* pv. *phleipratensis* (Lane 14), *X. c.* pv. *vasculorum* (Lane 15), and *X. c.* pv. *vesicatoria* (Lane 16).

perform. Different gels can be compared to each other more easily after PAGE and RFLP than REA. A major disadvantage of these techniques is the requirement for expensive equipment and supplies that are often unavailable in diagnostic clinics. One limitation of the RFLP technique, the use of radioactive materials, could be avoided by using nonradioactive biotin-labeled probes (10). Identification of *X. c.* pv. *holcicola* is currently accomplished by testing pathogenicity to sorghum and maize (*Zea mays* L.) (1). However, pathogenicity based on host range may not be sufficient to accurately group strains into pathovars of *X. campestris* (3,8). Based on the results from the PAGE, REA, and RFLP, we conclude that each of these techniques could provide an alternative method to pathogenicity tests for comparison of *X. c.* pv. *holcicola*.

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