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Genomic Fingerprints of *Xanthomonas campestris* pv. *citri* Strains from Asia, South America, and Florida

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ABSTRACT

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Genomic DNA was prepared from strains of *Xanthomonas campestris* pv. *citri* isolated in seven countries and in Florida. After the DNA was digested with restriction endonuclease *Eco* R1, the fragments were separated by polyacrylamide gel electrophoresis and the resulting genomic DNA fingerprints were compared. Nine Asiatic or A group strains were indistinguishable by this technique. Likewise, four cancrosis B group strains were also indistinguishable from each other. However, the A and B groups were clearly differentiated by this technique. The fingerprint of a single strain from Mexico, associated with citrus bacteriosis disease was, however, indistinguishable from that of the B group strains. In contrast to the apparently conserved genomic fingerprints observed with the A and B groups of strains, the strains isolated from diseased citrus in Florida (Group E) showed a wide variety of genomic fingerprints. These fingerprints varied not only among separate disease outbreaks but also within single outbreaks. These results are inconsistent with the idea that a new strain of *X. c.* pv. *citri* has been recently introduced into Florida but support the idea that the *X. campestris* strains isolated from Florida citrus are samples of an endemic flora.

Additional key words: citrus canker, restriction endonuclease fingerprinting.

Bacterial strains that belong to the same species are distinguishable by phage typing (6) or by serological analysis of antigenic determinants localized in the capsular polysaccharide and outer membrane protein (5,9), the lipopolysaccharide (10), or heat stable somatic antigens (6). Monoclonal antibodies of high specificity are also used to distinguish strains of *Xanthomonas campestris* pv. *campestris* (Pammel) Dawson (1). The variation in plasmid DNA content among bacterial strains has also been used as a distinguishing characteristic (7,8).

The distribution of restriction endonuclease cleavage sites in a bacterial genome is unique, stable, clonally inherited, and can be directly observed. These properties make this an ideal character to use in the study of the epidemiology of bacterial diseases. After extraction of the genomic DNA and digestion to completion with a specific restriction endonuclease and separation of the resulting fragments through either an agarose or a polyacrylamide gel, ethidium bromide stained bands can be viewed with an ultraviolet light. The resulting complex pattern of bands, the genomic fingerprint, can be directly compared with that of other strains and photographed to provide a permanent record. This approach has been successfully used to study the epidemiology of an outbreak of cholera caused by *Vibrio cholerae* Pacini (12), outbreaks of enteritis caused by *Campylobacter jejuni* Veron & Chatelain (5,11), and an outbreak of meningococcal disease in Norway caused by *Neisseria meningitidis* (Albrecht & Ghon) Murray (4,14). The technique has also been used to distinguish among strains of *Frankia* Brunchorst that were morphologically indistinguishable (2). A similar technique was originally developed for the analysis of eukaryotic genomes (18).

The recent outbreak in Florida (17) of an apparently new form of citrus bacterial canker disease, CBCD, caused by *X. c.* pv. *citri* (Hasse) Dye was studied using this technique. Several forms of CBCD have been previously described based on host range, geographical distribution, phage typing, and plasmid analysis of bacterial isolates. The bacterial strains that cause these forms of CBCD have been placed in four groups: A for Asiatic CBCD, B for cancrosis B in Argentina primarily, C for Mexican lime cancrosis in Brazil, and D for Mexican bacteriosis in Mexico (6). Isolates of *X. c.* pv. *citri* obtained from diseased citrus in Florida have been placed in group E, based on geographical origin, primary host plants affected, and symptomotology. Strains belonging to group E do not produce raised lesions on diseased plants as do all strains belonging to groups A–D, but instead produce flat, water-soaked lesions of various sizes (17).

MATERIALS AND METHODS

Bacterial strains and extraction of genomic DNA. For comparative purposes, genomic fingerprints were prepared from nine bacterial strains of the A or Asiatic group, four strains of the cancrosis B group from Argentina, and one strain of the citrus bacteriosis pathogen from Mexico (Group D). Fingerprints were also obtained from 16 strains of the E group received from the Department of Plant Industry (DPI), Gainesville, FL. (Table 1).

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Cultures of *X. c.* pv. *citri* were stored at 4 C under sterile mineral oil on slants of Wakimoto's semisynthetic potato medium (13). Colonies on LB agar (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of Bacto agar per liter of deionized water) were used to start 10-ml liquid LB cultures in 50-ml flasks. Two cultures were used per strain and were grown for 18 hr with gentle rotary shaking at 27 C. Genomic DNA was prepared as follows (3). The pooled 20-ml culture was centrifuged (10 min at 10,000 *g*) and the pellet was resuspended in 10 ml of PBS (20 mM potassium phosphate buffer, pH 6.9, which contained 150 mM NaCl). After a second centrifugation the pellet was resuspended in 5 ml of 50 mM Tris, pH 8.0, which contained 50 mM EDTA. Eggwhite lysozyme was added to a final concentration of 1 mg/ml and the tubes were incubated at 0 C for 30 min. One milliliter of a freshly prepared lysing solution (0.5% sodium dodecyl sulfate, 50 mM Tris/Cl, pH 7.5, 400 mM EDTA, and 1 mg/ml of pronase) was added to each tube, which was incubated at 50 C until the suspension cleared. The lysate was extracted with an equal volume of Tris buffer-saturated phenol (pH 7.8). After centrifugation (9,000 *g* for 10 min) the aqueous supernatant was transferred to a clean tube and sodium acetate was added to 0.3 M. After addition of two volumes of ethanol and mixing by inversion, the nucleic acids were removed by spooling onto a glass pipette and dissolved in 3 ml of TE (10 mM Tris/Cl, pH 8.0, 1 mM EDTA) containing RNase A (50 μg/ml). After 30 min at 37 C the solution was extracted with an equal volume of chloroform and the DNA was spooled out of the solution by a second ethanol precipitation. The DNA was dissolved in a minimal volume of TE and stored at 4 C until used. The concentration of DNA in the sample was estimated spectrophotometrically.

TABLE 1. Strains of *Xanthomonas campestris* pv. *citri* fingerprinted in this study

Strain designation			
Lab[a]	Source[b]	Origin[c]	Group[d]
XC59	IBBF-164	Brazil	A
XC62	6501	Japan	A
XC63	7801	Japan	A
XC91	XCC4B	Argentina	A
XC92	XCC64B	Argentina	A
XC98		Yemen	A
XC97		Yemen	A
XC100		Pakistan	A
XC99		Pakistan	A
XC64	B-4(LMLB7)	Argentina	B
XC69		Argentina	B
XC93	XCC83B	Argentina	B
XC94	XCC85B	Argentina	B
XC90		Mexico	D
F1	P843048-1	Florida 1	E
F2	P843048-2	Florida 1	E
F3	P843081-1	Florida 1	E
F4	P843081-2	Florida 1	E
F5	P843162-1	Florida 2	E
F29	X854600-R1	Florida 3	E
F30	X854600-R	Florida 3	E
F49	X854600-1	Florida 3	E
F94	X856774-3	Florida 4	E
F95	X857685-1	Florida 4	E
F96	X858893-3	Florida 4	E
F97	X859510-1	Florida 4	E
F98	X8511454-2	Florida 4	E
F99	X8511520-1	Florida 4	E
F128	B7	Florida 4, from weeds	
F130	B8	Florida 4, from weeds	

[a] Designations used in the Fruit Laboratory, Horticultural Science Institute, Beltsville Agricultural Research Center, Beltsville, MD.
[b] Designations used by the original source of the strains.
[c] Numbers following word "Florida" in column 3 refer to separate diseased nurseries from which these strains were independently isolated.
[d] Groups A and B were described in reference 6. Group D includes strains of the citrus bacteriosis pathogen of Mexico. All E group strains were isolated and confirmed to be pathogenic by the Department of Plant Industry, Tallahassee, FL.

Preparation of genomic fingerprints. Each restriction endonuclease digestion contained 3–5 μg of DNA and 20 units of enzyme *Eco* R1. Reaction volumes varied between 35 and 55 μl and buffer conditions were those recommended by the supplier. Incubation was at 37 C for 4 hr. Samples were loaded on a 1.5-mm-thick, 14-cm-long, vertical 5% polyacrylamide gel (15), and fragments were separated by electrophoresis at 14 mA constant current for 14 hr in TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). During electrophoresis, the voltage increased from 50V to 90V. Gels were stained with ethidium bromide (2 μg/ml) for 60 min then photographed on a transilluminator (Spectroline) using both an orange and a yellow filter and Polaroid type 55 high contrast film. Genomic fingerprints were compared using the photograph or with the negative and the aid of a photographic enlarger.

Source of reagents. Nutrient media were from Difco, Detroit, MI. Egg white lysozyme, RNAse A, buffers, and salts were from Sigma Chemical Co., St. Louis, MO. Acrylamide reagents were from Bio-Rad Laboratories, Richmond, CA. Pronase was from Calbiochem-Behring, La Jolla, CA. Molecular biology grade phenol was from International Biotechnologies, Inc. *Eco* R1 and DNA size standards were from Bethesda Research Laboratories, Gaithersburg, MD.

RESULTS

The fragments generated by *Eco* R1 digestion of genomic DNA were resolved into reproducible patterns of bands, in at least three gels, after electrophoresis through 5% polyacrylamide gels. The pattern of bands produced by individual strains from the A and B groups of strains fell into two groups. The nine A group strains were extremely similar by this technique (Fig. 1), as were the four B group strains (Fig. 2). However, the A and B group fingerprints were clearly different (Figs. 1 and 2). The fingerprint of the single available strain of the D group matched that of the B group of strains (Fig. 2).

In contrast to the results observed with the collection of strains from the A and B groups of *X. c.* pv. *citri*, the collection of E group strains associated with the Florida form of CBCD did not share a common fingerprint (Figs. 3 and 4). Strains F1–F5 had a common fingerprint that was very different from the one shared by strains F29, F30, and F49 (Fig. 3). The genomic fingerprints also varied among independent isolates from the same disease occurrences (Fig. 4). For example, seven different fingerprints were observed with strains F94–F99 and F128 and F130, all of which were

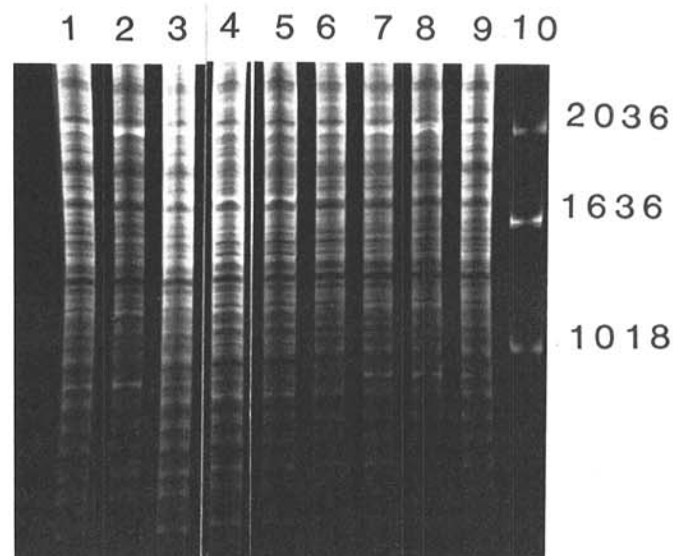

Fig. 1. Genomic fingerprints of A group strains of *Xanthomonas campestris* pv. *citri*. Lane 1, XC59 (Brazil); Lane 2, XC62; Lane 3, XC63 (Japan); Lane 4, XC99; Lane 5, XC100 (Pakistan); Lane 6, XC97; Lane 7, XC98 (Yemen); Lane 8, XC91; Lane 9, XC92 (Argentina); Lane 10 contains size standards marked in base pairs in the margin.

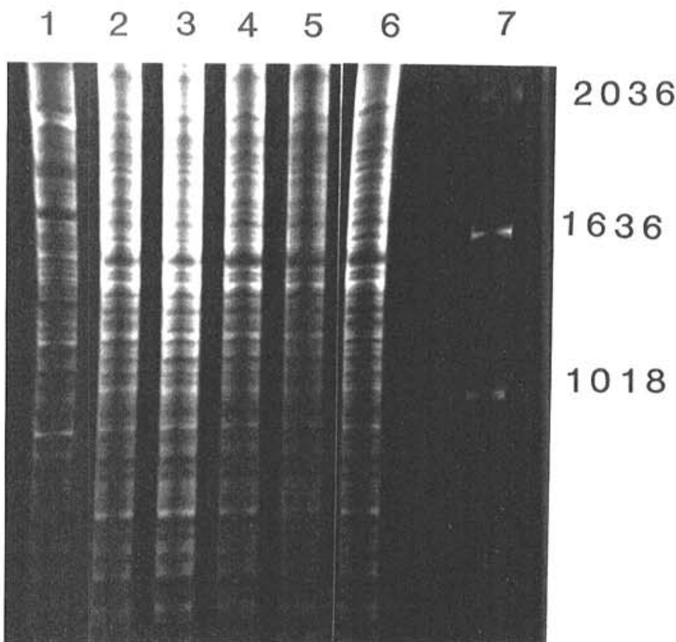

Fig. 2. Genomic fingerprints of B group strains *Xanthomonas campestris* pv. *citri* (lanes 2–5) compared with an A strain (lane 1) and a D strain (lane 6). Lane 1, XC62 (Japan); Lane 2, XC64; Lane 3, XC69; Lane 4, XC93; Lane 5, XC94 (Argentina); Lane 6, XC90 (Mexico); Lane 7 contains size standards marked in base pairs in the margin.

independently isolated from the same diseased nursery. Likewise two distinct fingerprints were observed from a third disease outbreak, represented by strains F66, F67, and F68 (data not shown). Based on these results, it must be concluded that several

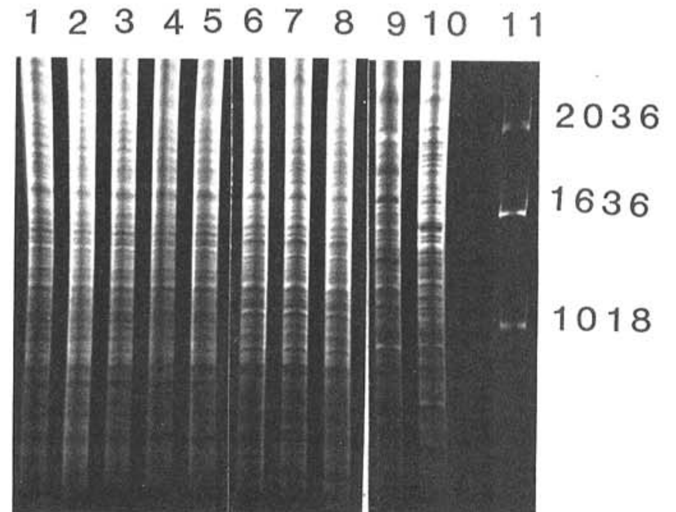

Fig. 3. Genomic fingerprints of E group strains from three nurseries (lanes 1–8) and an A and a B strain (lanes 9–10). Lane 1, F1; Lane 2, F2; Lane 3, F3; Lane 4, F4 (nursery 1); Lane 5, F5 (nursery 2); Lane 6, F29; Lane 7, F30; Lane 8, FY 9 (nursery 3); Lane 9, XC62; Lane 10, XC93; Lane 11 contains size standards marked in base pairs in the margin.

Fig. 4. Genomic fingerprints of E group strains from nursery 4, isolated from diseased citrus (lanes 1, 2, 4, 5, 7, 8) and from weeds (lanes 3, 6, 9, 10). Lane 1, F94; Lane 2, F95; Lane 3, F128; Lane 4, F96; Lane 5, F97; Lane 6, F130; Lane 7, F98; Lane 8, F99; Lane 9, F128; Lane 10, F130. Lane 11 contains size standards marked in base pairs in the margin.

strains of *X. c.* pv. *citri* are associated with the Florida outbreak of CBCD primarily in citrus nurseries. These strains can be distinguished from each other on the basis of their genomic fingerprints.

DISCUSSION

The A and B groups of *X. c.* pv. *citri* can be distinguished by their *Eco* R1 genomic fingerprints; strains within a group are not distinguishable by this technique. Strains within a group are not necessarily identical, however, since a great deal of mutation would go undetected by this technique. The results of the genomic fingerprinting support the grouping of strains into groups A and B, which was done previously using separate criteria, including geographic distribution, primary host affected, phage typing, and serology (6).

The distribution of *Eco* R1 or other restriction endonuclease cleavage sites are expected to be very stable in bacterial genomes. The nine A group strains fingerprinted (Fig. 1) had been isolated in six countries and had been in culture collections for periods of time ranging from 2 to 12 yr. The *Eco* R1 fingerprints were extremely similar, strongly suggesting a clonal descent (16) for these strains. A 10th A group strain was obtained from the DPI in Florida. The fingerprint of this strain, which was isolated in October of 1985 and which may have originated in Thailand, was indistinguishable from those of the other A group of strains (data not shown). A different genomic fingerprint was shared by the four B group strains, which had been isolated over a period of years from Corrientes Province, Argentina, also suggesting a clonal descent for these strains.

The fingerprint data also suggest a clonal relationship between the single available D strain (Mexico) and the B group (Fig. 2). The single available C strain was similar to the B group (data not shown). It would be very worthwhile to collect more strains causing Mexican bacteriosis (D) and Mexican lime cancrosis (C) to determine if these relationships can be confirmed.

The indistinguishable genomic fingerprints of strains F1–F5 isolated from two different diseased nurseries strongly suggests a clonal relationship (16) for these strains, which is different from that of strains F29, F30, and F49, which are from a third diseased nursery (Fig. 3). Strains of *X. c.* pv. *citri* isolated from nursery weeds shared a common fingerprint with each other, but not with any of six strains isolated previously from diseased citrus in the same nursery (Fig. 4). This latter nursery, number 4 (Table 1) seems to have a particularly heterogeneous mixture of *X. c.* pv. *citri* strains, although similar results were obtained for several other nurseries (data not shown).

The diversity of genomic fingerprints obtained from the E group of strains associated with the Florida form of CBCD is inconsistent with a recent introduction of a single strain into Florida. If that were the case, a single identifiable genomic fingerprint would be expected. Instead, the genomic fingerprints of the E group of strains were found to vary among (Fig. 3) and even within (Fig. 4) disease occurrences. Because a variety of fingerprints is observed within the E group, there is probably a population of *X. c.* pv. *citri* "E" strains associated with CBCD in Florida.

The possibility that variations in plasmid DNA content (8) were responsible for the heterogeneity of the genomic fingerprints of the Florida strains was examined. Strains F1–F5 had indistinguishable genomic fingerprints (Fig. 3) yet only strain F1 contained a detectable plasmid (data not shown). Strains F94, F95, and F97 all lacked detectable plasmids (data not shown) yet their genomic fingerprints varied (Fig. 4). Also A group strains Xc62 and Xc63 are known to have different plasmid profiles (7) and yet had indistinguishable genomic fingerprints (Fig. 1). Thus, there was no evidence that plasmid DNA was contributing to the genomic fingerprints.

Given the diversity of genomic fingerprints observed, it is difficult to envision a genetic event suddenly giving rise to a population of bacterial strains that share the ability to attack citrus but do not share a common genomic fingerprint. However, integration of plasmids into the genome, such as has been described for *Pseudomonas syringae* pv. *phaeseolicola* (19) would likely alter the genomic fingerprints of the strain. It is possible that the E group of strains of *X. c.* pv. *citri* represent a sample of an endemic flora, such as has been found in Norway for *N. meningitidis*, where many different genomic fingerprints were found among strains of the same serotype (14). It is worth noting in this connection that nearly all isolations of strains of the E group are from lesions found on a single cultivar (Swingle) of citrus. This cultivar has only very recently become popular enough to have large acreages of nursery plantings devoted to it. The possibility exists that the E group of strains that have been isolated in Florida may be more accurately described as epiphytes on other *Citrus* spp. or as epiphytes or pathogens on other noncitrus hosts, which share the ability to cause disease on this newly popular citrus cultivar. This possibility needs to be investigated further.

The technique of genomic fingerprinting could prove to be an invaluable aid to the study of the epidemiology of diseases caused by phytopathogenic bacteria, or in any application that required rapid and precise strain identification.

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