Plasmid DNA Fingerprints Distinguish Pathotypes of *Xanthomonas campestris* pv. *citri*, the Causal Agent of Citrus Bacterial Canker Disease

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ABSTRACT

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Plasmid DNA was isolated from 54 strains of *Xanthomonas campestris* pv. *citri*, associated with different forms of citrus bacterial canker disease (CBCD). The number of plasmids per strain varied from one to five. A total of 24 plasmid bands with sizes from 7 to 100 kilobases (kb) were identified. Strains that had identical plasmid profiles were generally associated with the same form of CBCD. After digesting the plasmid DNA with each of three restriction endonucleases, 87 fragments with different sizes from about 1 to 30 kb were visualized. Strains belonging to a specific CBCD group shared plasmid DNA fragments of similar sizes. Dendrograms derived from plasmid DNA fingerprint analyses allowed us to clearly distinguish A, B, and C pathotypes of *X. c. citri*. The strain Xc90, associated with bacteriosis of Mexican lime in Mexico (CBCD-D) was not clearly distinguishable from strains associated with cancrosis B (CBCD-B) from Argentina and Uruguay. Plasmid DNA fragments specifically associated with some groups of strains were identified. A *Bam*HI fragment from a CBCD-A strain was used as a hybridization probe. A strong signal was recorded in all CBCD-A strains studied. Weaker hybridization signals were observed with one or two high molecular weight bands in all CBCD-B strains studied. All three type C strains had a band of slightly smaller size than the probe, but which hybridized only very weakly. Strain Xc70 also had a homologous larger band similar in size to one found in the CBCD-B strains. Hierarchical cluster analysis of the RFLP data from the plasmid DNA revealed phenetic clusters strikingly similar to those obtained previously from analysis of genomic DNA, lending support to the concept of balanced co-evolution of plasmid and chromosomal genomes.

Citrus bacterial canker disease (CBCD) is caused by *Xanthomonas campestris* pv. *citri* (Hasse) Dye (8). Several forms of the disease are recognized. It is currently believed that at least three forms of CBCD (A, B, and C cankers) occur around the world (3,34) and are induced by variants of the same causal agent. These variants are primarily distinguished by their geographical origin and their host range. Some pathogenic strains of *X. campestris* may be associated with citrus bacteriosis in Mexico, and they were described as the D form of CBCD (3,17). Another group of strains of *X. campestris*, which is genomically heterogenous and different from *X. c. citri*, is associated with citrus bacterial spot disease (CBSD) in Florida (10,16,17,31). A proposal to elevate the strains of *X. c. citri* associated with CBCD-A to *X. citri* and to rename the strains associated with CBCD-B/C/D *X. c. aurantifolii* and the CBSD strains *X. c. citrumelo* (11) was contested (38). Thus, the taxonomic position of these strains is controversial.

Several laboratory techniques (e.g., biochemical tests and metabolic fingerprinting, phage typing, serology using polyclonal or monoclonal antibodies, genomic fingerprinting, isozymes, RFLP analyses, and plasmid DNA analyses) have been used to characterize the different strains of *X. c. citri* (1,2,4,5,10,14, 16,17,21,22).

Plasmids occur in strains of *X. campestris* belonging to numerous pathovars (i.e., *campestris*, *cyamopsidis*, *dieffenbachiae*, *glycines*, *hederae*, *malvacearum*, *manihotis*, *phaseoli*, *pruni*, *vesicatoria*, *vignicola*, and *vitians* (9,15,22,29,33). Plasmid content and RFLP analysis of plasmid DNA were reported to be useful for pathovar identification of strains of *X. campestris* (23).

Indigenous plasmids have been reported in all strains of A, B, and C types of *X. c. citri* associated with different forms of CBCD (4). No specific phenotypic functions were associated with these plasmids. Although it was possible to differentiate types of CBCD based on plasmid profiles of the associated strains, plasmid profiles were also reported to be variable among strains belonging to the same type (4). These results were different from those of Stripecke and Rosato (35) who found plasmid profiles to be highly stable in strains of Asiatic form of CBCD using a limited number of strains, the origin of which was not described. Recently, Ulaganathan and Mahadevan (37) reported that some strains of *X. c. citri* from India did not carry plasmids.

The purposes of this study were to verify that plasmid DNA occurs in *X. c. citri*, to determine the range in plasmid DNA variation (i.e., number and size), to estimate the relationships among plasmids of strains of *X. c. citri* associated with the A, B, C, and D forms of CBCD, and to evaluate the usefulness of these plasmids in epidemiological studies. Furthermore, a fragment of plasmid DNA that was characteristic of subgroup A of *X. c. citri* was cloned in *Escherichia coli* and used as a hybridization probe. Preliminary experiments indicate that it can be used to distinguish forms of *X. c. citri* from each other.

MATERIALS AND METHODS

Bacterial strains. Information on 54 strains of *X. c. citri* included in this study is provided in Table 1. All of these strains produced typical "cankerlike" lesions after artificial inoculation of Mexican lime and/or grapefruit leaves. Cultures were checked for purity on LPGA (7 g of yeast extract, 7 g of bactopeptone, 7 g of glucose, 15 g of agar, 1,000 ml of deionized water, pH 7.2) plates before plasmid DNA isolation.

Plasmid DNA isolation, endonuclease digestion, and electrophoresis. A slightly modified version of the Kado and Liu (19) method was used to isolate plasmid DNA. Cultures were grown for 24 h (48 h for the B/D strains) at 28 C on LPA medium (7 g of yeast extract, 7 g of bactopeptone, 15 g of agar, 1,000 ml of distilled water, pH 7.2) and then were transferred to flasks containing 20 ml of modified Wilbrink's broth (5 g of bactopeptone, 2.5 g of sucrose, 0.5 g of K_2HPO_4, 0.25 g of $MgSO_4$, 1,000 ml of distilled water). CBCD-A strains were shaken for 18 h at 28 C; CBCD-B/C/D strains were shaken for 24 h at

the same temperature. Cells were collected by centrifugation (Beckmann JA 20 rotor, 10,000 rpm, 15 min, 4 C; Beckman Instruments, Fullerton, CA). The pellets were resuspended in 2 ml of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed using 4 ml of Kado and Liu's lysing solution. Samples were incubated for 15 min at room temperature and then for 2 h at 65 C, which was the time required to completely degrade the genomic DNA. Samples were extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and were centrifuged as described above. One milliliter of sodium acetate (3 M, pH 5.2) was added to 5 ml of the aqueous phase containing the plasmid DNA. Samples were incubated on ice for 10 min and then were centrifuged as described above. Plasmid DNA was precipitated by adding two volumes of cold 95% ethanol and incubating for 1 h at −20 C. DNA obtained after centrifugation (13,000 rpm, 15 min, 4 C) was washed with 5 ml of 70% ethanol. Pellets obtained after centrifugation were resuspended in 500 μl of TAE buffer and were stored at 4 C.

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

Strain	Origin	Host	Year of isolation
CBCD-A			
Xc91	Argentina		
Xc92	Argentina		
Xc101	Guam		
Xc102	Guam		
Xc164	India		1988
Xc165	India		1988
Xc166	India		1988
Xc168	India		1988
Xc169	India		1988
Xc62	Japan		1978
Xc63	Japan		1978
Xc87	Japan	*Citrus reticulata*	1984
CFBP[a] 2854	Japan		
CFBP 2855	Japan		1962
Xc124	Korea	*C. reticulata*	1987
Xc126	Korea	*C. reticulata*	1987
Xc127	Maldives Islands	*C. aurantifolia*	1987
Xc128	Maldives Islands	*C. aurantifolia*	1987
Xc130	Maldives Islands	*C. aurantifolia*	1987
Xc131	Maldives Islands	*C. aurantifolia*	1987
Xc132	Maldives Islands	*C. aurantifolia*	1987
Xc133	Maldives Islands	*C. aurantifolia*	1987
Xc134	Maldives Islands	*C. aurantifolia*	1987
Xc135	Maldives Islands	*C. aurantifolia*	1987
Xc136	Maldives Islands	*C. aurantifolia*	1987
Xc137	Maldives Islands	*C. aurantifolia*	1987
Xc115	New Zealand[b]	*C. limon*	1956
Xc100	Pakistan		
Xc156	Pakistan	*C. sinensis*	1988
Xc158	Pakistan	*C. sinensis*	1988
Xc159	Pakistan	*C. sinensis*	1988
Xc160	Pakistan	*C. sinensis*	1988
Xc162	Pakistan	*C. sinensis*	1988
Xc163	Pakistan	*C. sinensis*	1988
Xc138	Philippines		1988
Xc139	Philippines		1988
Xc141	Philippines		1988
Xc142	Philippines		1988
Xc144	Philippines		1988
Xc145	Philippines		
Xc81	Uruguay	*C. paradisi*	1983
Xc98	Yemen		
CBCD-B			
Xc64	Argentina	*C. limon*	1979
Xc69	Argentina	*C. limon*	1979
Xc93	Argentina	*C. limon*	
Xc94	Argentina	*C. limon*	
Xc96	Argentina	*C. limon*	
Xc148	Argentina	*C. limon*	1988
Xc80	Uruguay	*C. limon*	1983
Xc84	Uruguay	*C. limon*	1984
CBCD-C			
Xc70	Brazil	*C. aurantifolia*	
CFBP 2905	Brazil	*C. aurantifolia*	1981
CFBP 2906	Brazil	*C. aurantifolia*	1981
CBCD-D			
Xc90	Mexico	*C. aurantifolia*	

[a]CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Station de Pathologie Végétale, Rue G. Morel, 49070 Beaucouze, France.
[b]Type strain NCPPB409.

Extracted DNAs were separately digested with restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII, per manufacturer's specifications (Gibco/BRL, Gaithersburg, MD). Plasmids and plasmid fragments were separated by size, using agarose gel electrophoresis (0.6 or 1.0% SEAKEM LE agarose, Marine colloids, Rockland, ME) at 5 V/cm in TAE buffer (pH 8.0). They were visualized with ultraviolet light (302 nm) after staining gels for 30 min in 1 mg/L of ethidium bromide. Pictures were taken, using a yellow filter with Polaroid films (type 52 or 55). Plasmid sizes in kilobases (kb) were estimated by linear regression analyses of log relative mobility versus log kb by using plasmid DNA of *Erwinia stewartii* SW2 as the reference marker (7). Sizes of DNA restriction endonuclease fragments were estimated using lambda phage digested with *Hin*dIII. All the experiments were done at least twice for each strain.

Molecular cloning and Southern hybridizations. For cloning experiments, plasmid DNA samples were completely digested with restriction endonuclease *Bam*HI following the manufacturer's directions. Fragments were resolved by agarose gel electrophoresis, and the desired fragments were eluted with glassmilk (Bio-101, Inc., La Jolla, CA) before ligation into vector pUC9 (27). Competent *E. coli* cells were transformed with the ligation mixtures (strain JM103: *lac*, *pro*, *sup*E, *str*A, *end*A, *sbc*B15, *hsd*R4, F′ *tra*D36, *pro*AB, *lac*I^R, ZM15) and plated on LB medium (10 g of bactotryptone, 5 g of yeast extract, 5 g of NaCl, 15 g of agar, 1,000 ml of deionized water, pH 7.5) supplemented with ampicillin at 200 mg/L, Bluo-Gal (Gibco/BRL) at 60 mg/L, and IPTG at 12 mg/L. White colonies were screened for inserts with the boiling mini-prep method (25).

For Southern blotting, genomic DNA from strains of *X. c. citri* was isolated and digested with endonuclease *Bam*HI (4 U/μg) for 3 h at 37 C. Aliquots of 500 ng were electrophoresed at 20 V for 16.5 h in 0.8% agarose in TAE buffer. Capillary transfers were performed to photogene membranes (Gibco/BRL), which were fixed by baking at 80 C for 90 min. Hybridizations were performed at 68 C overnight in a solution that contained 6× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.01 M EDTA, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml of denatured salmon sperm DNA (25). Two posthybridization stringency washes were with 0.1× SSC, 0.5% SDS at 68 C for 30 min each. The probe used was a cloned 4.2-kb *Bam*HI fragment from strain Xc62 (A type), which was removed from the vector by endonuclease digestion, agarose gel electrophoresis, and elution with glassmilk before labeling. The probe was used at about 20 ng/ml. Labeling with biotin by nick translation and detection was done using the photogene chemiluminescent detection kit (Gibco/BRL). Exposure of Hyperfilm (Amersham Corp., Arlington Heights, IL) was for 2–5 min at 23 C.

Hierarchical cluster analysis. Plasmid DNA fingerprint data were used for hierarchical cluster analysis. Presence (coded 1) or absence (coded 0) of each fragment was recorded. The Sokal and Sneath coefficient (32) was calculated for each pair of strains, using the following equation:

$$d_{s.s} = 1 - \frac{n_{xy}}{n_{xy} + 2(n_x + n_y)} \quad (1)$$

in which n_{xy} is the number of fragments shared between the pair of strains, and n_x and n_y respectively, are the number of fragments present in strain x and absent in strain y, and the number of fragments present in strain y and absent in strain x. This coefficient

was compared to the Nei and Li coefficient (28) that was calculated by the following equation:

$$d_{n.l} = 1 - \frac{2n_{xy}}{(n_x + n_y)} \quad (2)$$

in which n_{xy} is the number of fragments shared between the pair of strains, and n_x and n_y respectively, are the number of fragments present in strain x and the number of fragments present in strain y. The coefficients calculated were averages of three observations (fingerprinting with three restriction endonucleases: *Bam*HI, *Eco*RI, and *Hin*dIII). Hierarchical cluster analysis was done by average linkage clustering (13) using the ADDAD program on a Data General mainframe computer.

RESULTS

Characterization of plasmid DNAs. All the strains of *X. c. citri* included in this study contained at least one to five plasmids. From 54 strains, 24 plasmids ranging in size from 7 to 100 kb were visualized (Table 2). A 30-kb plasmid was the most commonly detected (in 36 strains). Plasmids with sizes of 8, 9, 28, 38, 49, 54, 61, 65, 68, 76, 80, 90, and 100 kb occurred only in strains associated with the Asiatic form of CBCD. Plasmids in the size range of 32–36 kb were present in all type B, C, and D strains, except the type B strain Xc64. Plasmids in this size range were not detected in the 42 type A strains tested (Table 2). A 7-kb plasmid occurred only in one strain associated with cancrosis C. Strains that had indistinguishable plasmid profiles were generally associated with the same form of CBCD (Table 2). Plasmid profiles of strains Xc80 and Xc90 were similar, although one strain (Xc80) was isolated in Uruguay and was associated with CBCD-B, and the second one (Xc90) was isolated in Mexico and was associated with bacteriosis of Mexican lime in Mexico (CBCD-D). Agarose gels of 0.6% were more suitable than 1.0% agarose gels to separate plasmids with similar sizes (e.g., 32- and 33-kb plasmids in strains Xc80 and Xc90). These two plasmids were seen as only one band when separated in 1.0% agarose gels.

After digesting the plasmid DNAs with three restriction endonucleases, 87 fragments with sizes from about 1 to 30 kb were visualized. Strains belonging to each CBCD group shared DNA fragments of the same size. No fragment was common to all the studied strains. Two fragments obtained after digesting plasmid DNA with *Bam*HI (3.7 and 1.0 kb) were visualized only in strains associated with cancrosis C. One fragment (3.9 kb), obtained after digesting DNA with *Eco*RI, was found in all the B strains. It was also visualized in the strain associated with bacteriosis in Mexico (Xc90), but was not detected in strains associated with Asiatic canker and cancrosis C. One *Hin*dIII fragment of 15.9 kb occurred in all strains associated with B, C, and D forms of CBCD, but was not present in strains associated with CBCD-A. No fragment was common to all the strains associated with CBCD-A. However, a 4.2-kb *Bam*HI fragment and a 2.7-kb *Eco*RI fragment occurred in 85.2 and 90.7%, respectively, of the studied CBCD-A strains.

TABLE 2. Plasmid profiles of strains of *Xanthomonas campestris* pv. *citri* associated with different forms of citrus bacterial canker disease

Strain	Number of plasmids	Sizes (kb) of plasmids																							
		100	90	80	76	68	65	61	54	49	45	43	40	38	36	34	33	32	30	28	26	18	9	8	7
CBCD-A																									
Xc92[a]	3	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc101[b]	1	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xc102	2	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Xc164	3	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
Xc165	2	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Xc166	5	−	−	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	−
Xc168	4	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−
Xc169	4	−	−	−	−	−	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Xc62[c]	2	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc63	2	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
2855	3	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc127[d]	3	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc130[e]	4	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Xc115	2	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xc100	3	−	−	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xc156[f]	3	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−
Xc163	3	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc145	1	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xc98	3	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
CBCD-B																									
Xc64	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Xc69	3	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	+	−	−	−	−	−	−
Xc93	3	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−
Xc94	3	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−
Xc96[g]	4	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−
Xc80	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
Xc84	4	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−
CBCD-C																									
Xc70	2	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
2905	4	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	+
2906	3	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−
CBCD-D																									
Xc90	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−

[a] Strains Xc92 and Xc81 had identical plasmid profiles.
[b] Strains Xc101 and 2854 had identical plasmid profiles.
[c] Strains Xc62, Xc87, Xc91, Xc124, Xc126, Xc138, Xc139, Xc141, Xc142, and Xc144 had identical plasmid profiles.
[d] Strains Xc127, Xc128, Xc131, Xc132, Xc133, Xc134, Xc135, and Xc137 had identical plasmid profiles.
[e] Strains Xc130 and Xc136 had identical plasmid profiles.
[f] Strains Xc156, Xc158, Xc159, Xc160, and Xc162 had identical plasmid profiles.
[g] Strains Xc96 and Xc148 had identical plasmid profiles.

All strains that shared a common plasmid profile (uncut DNA) had similar fingerprints with the three restriction endonucleases used, except strains Xc80 and Xc90. The single 30-kb plasmid present in strain Xc64 (CBCD-B) was cut into four fragments (14.4, 9.6, 6.2, and 3.9 kb) when using *Eco*RI. These fragments were detected in all the B strains in which this plasmid occurred (Xc64, Xc69, Xc96, and Xc148), but were not present in strains associated with CBCD-A, which also carried a plasmid that had the same electrophoretic mobility. Strains that carried two plasmids of 68 and 30 kb had fingerprints identical to strains carrying three plasmids, respectively, of 90, 68, and 30 kb. Thus, it may be possible that the 90-kb plasmid is a chimeric of 68- and 30-kb plasmids. These results demonstrate the superior information obtained by endonuclease digestion of plasmid DNA.

Only minor differences in the dendrograms produced by the two coefficients were observed. The dissimilarity coefficient of Nei and Li was used to cluster the 54 strains into two main groups (Fig. 1). One group contained all the strains associated with CBCD-A (Asiatic canker), and the second one contained the strains associated with B, C, and D forms of CBCD. Within the B/C/D group, two subgroups (C strains vs. B/D strains) occurred. It was not possible to differentiate clearly the CBCD-B strains from the strain (Xc90) associated with citrus bacteriosis in Mexico (CBCD-D). Its plasmid DNA profile was similar to that of strain Xc80, which was isolated from lemon in Uruguay.

Polymorphisms occurred among strains within clusters. Within the C group, strains 2905 and 2906 were closely related, but are quite different from strain Xc70. Among strains of the B/D group, strain Xc64 was unique and thus distinguished from the other strains associated with CBCD-B. Two subgroups seem to occur within strains associated with A canker. Strains from Argentina, Korea, Maldives Islands, Pakistan, Philippines, Uruguay, Yemen, as well as most of the strains from Japan, composed the major subgroup. This subgroup also included the type strain (from New Zealand). The second subgroup consisted of strains Xc164, Xc166, and Xc169 (all from India). These strains were also associated with strain Xc100 (from Pakistan) when the Sokal and Sneath coefficient was used (not shown) and with strains Xc101 (from Guam) and 2854 (from Japan) when the Nei and Li coefficient was used (Fig. 1). Strain Xc165 from India was unique.

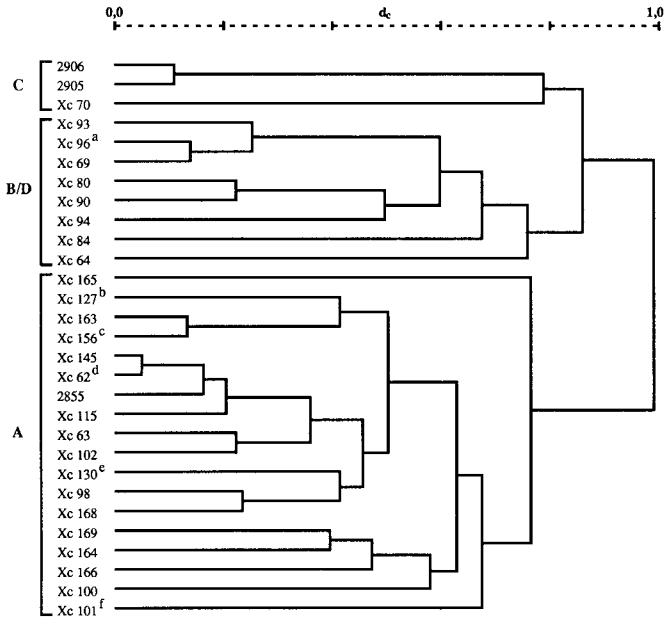

Fig. 1. Dendrogram of dissimilarity using the Nei and Li coefficient (28), clustered by the average linkage method, showing the relationships among strains of *Xanthomonas campestris* pv. *citri* associated with A, B, C, and D forms of CBCD based on plasmid DNA fingerprinting. [a]Strains Xc96 and Xc148 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII. [b]Strains Xc127, Xc128, Xc131, Xc132, Xc133, Xc134, Xc135, and Xc137 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII. [c]Strains Xc156, Xc158, Xc159, Xc160, and Xc162 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII. [d]Strains Xc62, Xc81, Xc87, Xc91, Xc92, Xc124, Xc126, Xc138, Xc139, Xc141, Xc142, and Xc144 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII. [e]Strains Xc130 and Xc136 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII. [f]Strains Xc101 and 2854 had identical fingerprints using the restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII.

A bimodal distribution of dissimilarity coefficients, which is a suitable criterion for identifying a good phenetic classification of groups of strains (24) was observed for the Nei and Li coefficient (Fig. 2), but not for the Sokal and Sneath coefficient (not shown). The utility of this criterion is intuitive; in this case, the coefficients cluster around two values, corresponding to two cophenetic clusters.

Southern hybridizations. A fragment indistinguishable from the 4.2-kb *Bam*HI fragment cloned from strain Xc62 was found in all type A strains studied (Fig. 3A). However, variation among strains was observed in single, higher molecular weight bands that hybridized weakly to this probe. Weaker hybridization signals were observed with one or two high molecular weight bands in all type B strains studied (Fig. 3B). All three type C strains shared a band of 3.6 kb, which hybridized only very weakly to the probe. Strain Xc70 also had a larger band similar to that in the type B strains (Fig. 3B).

DISCUSSION

Plasmids with sizes ranging from 7 to 100 kb were detected in all strains of *X. c. citri*, whatever their pathological group and origin. This confirms the conclusions of an earlier study (4). Our results disagree with those published by Ulaganathan and Mahadevan (37), who did not detect plasmids in strains of *X. c. citri* isolated in India. Two to five plasmids were visualized in the five Indian strains of *X. c. citri* included in our study. It is possible that the method used by these authors (37) was not suitable for *X. c. citri*, because they successfully isolated plasmids from other plant pathogenic bacteria.

Plasmid profiles were highly variable among strains of *X. c. citri*, because 24 plasmids with different sizes were identified. Some plasmids were found only in strains belonging to one pathotype. However, there was no plasmid that occurred in all the strains of a pathotype and that was absent in the strains of other patho-

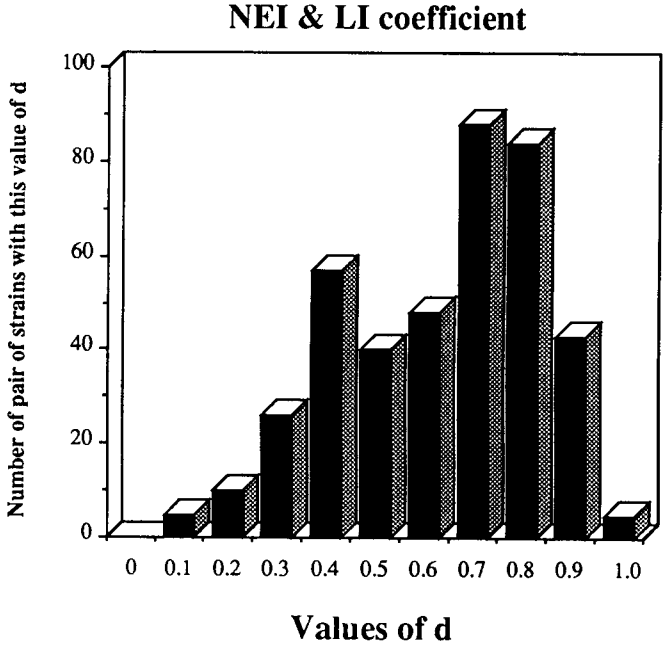

Fig. 2. Histogram showing the distribution of the Nei and Li dissimilarity coefficients (28).

types. This variation in plasmid profiles among strains confirms results published previously (4), but disagrees with results published by Stripecke and Rosato (35).

In this study, three plasmid bands were detected in strain Xc69 associated with cancrosis B in Argentina. Only two plasmids were reported in this strain in a previous study (4) in which 1% agarose gels were used. In our study, plasmids of 30 and 34 kb were seen as one wide band in 1% agarose, but were seen as two separate bands when using 0.6% gels. Plasmid contents of other strains studied by Civerolo (4) and in this study were identical.

After digesting the plasmid DNAs with restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII, 87 fragments with sizes from about 1 to 30 kb were visualized. Both dissimilarity coefficients used to create dendrograms from fingerprinting analyses revealed two main groups. One group is composed of all the strains associated with Asiatic canker, and the second group of the strains is associated with B, C, and D forms of CBCD. Within this second group, it is possible to distingulsh clearly strains associated with cancrosis C in Brazil (CBCD-C) from strains associated with cancrosis B in South America and with citrus bacteriosis in Mexico (CBCD-D). Consistent with previous analyses of genomic DNA (17), it is not possible to distinguish B and D forms of CBCD based on plasmid DNA fingerprints. The diversity of plasmid types observed in *X. c. citri* is not unique. Using plasmid DNA fingerprinting, diversity within *Pseudomonas syringae* pv. *pisi* was shown previously (20), similar to the diversity observed in this study within *X. c. citri.*

In the present study, the cloned DNA fragment hybridized to sequences in all tested strains of *X. c. citri.* Hybridization was however more intense to strains that belonged to subgroup A from which the probe was derived, and was produced to a 4.2-kb fragment, a fragment the same size as the probe (Fig. 3A). Sequences homologous to the cloned DNA probe were present on DNA fragments larger than 20 kb in all strains of *X. c. citri* belonging to subgroups B, C, and D (Fig. 3B). Thus, the sizes of DNA fragments with sequences homologous to the 4.2-kb *Eco*RI fragment distinguished strains belonging to subgroups A and B/C/D of *X. c. citri.*

A large number of dissimilarity coefficients can be found in the literature, and the dendrogram ultimately obtained depends to some extent on the choice of coefficient. We used two coefficients that are based on different logic to produce dendrograms from the same data: the Nei and Li coefficient doubles the weight of positive matches, and the Sokal and Sneath coefficient doubles the weight of mismatches. Clusters that do not change according to the chosen coefficient can be seen as strong clusters; clusters that change with the coefficient should be interpreted with caution. The clustering criterion used was the average linkage method in both cases.

Dendrograms obtained after computing the Nei and Li coefficient (Fig. 1) and the Sokal and Sneath coefficient (not shown) were almost identical. The dendrograms obtained were unique and did not depend on the order of strains in the data matrices. Both showed the same groups and subgroups. Classification of only a few strains of *X. c. citri* associated with Asiatic canker (Xc100, Xc101, 2854) was dependent on the dissimilarity coefficient used. In this case, we conclude that the Nei and Li coefficient was better than the Sokal and Sneath coefficient, because the ability of the Nei and Li coefficient to classify strains within the two main groups (A and B/C/D) appeared to be better than that of the the Sokal and Sneath coefficient. The bimodal distribution of dissimilarity coefficients obtained with the Nei and Li coefficient (Fig. 2) supports this view (24). Furthermore, dendrograms constructed using this dissimilarity coefficient with fingerprints obtained for each of the three restriction endonucleases (data not shown) are almost identical to the one shown in Figure 1. This suggests that this classification of the strains is not restriction endonuclease-dependent and that a similar classification would have been obtained using other enzymes.

Coplin (6) has suggested that indigenous plasmids in bacteria have co-evolved with their host's chromosome, so that together they constitute a balanced genome. A test for this hypothesis of co-evolution of plasmids and chromosomes would be to see if RFLP analysis of natural populations reveals similar phenetic clusters based on plasmid and chromosomal DNA. Significantly, the conclusions of our study are very similar to those obtained previously using total genomic DNA (16) and total genomic DNA probed with cloned chromosomal fragments (10,17,18), thus supporting the hypothesis of co-evolution of plasmid and chromosomal genomes. However in the present study, plasmid DNA fingerprinting revealed more polymorphism than did RFLP analyses of genomic DNA (17,18), suggesting that it may provide a more sensitive measure of diversity and provide a convenient technique for tracking strains in epidemiological studies or in quarantine applications. Plasmid DNA fingerprinting has also the advantage of being quicker to set up than RFLP analyses on total DNA, because it is possible to get good results with the mini-prep described in this study, and no recombinant DNA probes are required.

Specific plasmid DNA fragments were found in strains belonging either to the B/D group (3.9-kb *Eco*RI fragment), or the C group (3.7- and 1.0-kb *Bam*HI fragments). Two fragments (4.2-kb *Bam*HI and 2.7-kb *Eco*RI) occurred in most of the strains associated with Asiatic canker. A plasmid DNA fragment was previously used as a diagnostic probe for *X. c. phaseoli*, the causal agent of common bacterial blight of bean (12). This probe, which contained repetitive DNA sequences, detected *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans.* These results make the development of useful diagnostic probes for *X. c. citri* a possibility, as has been done for *P. s. phaseolicola* (30), *Clavibacter michiganensis* subsp. *michiganensis* (36), and pathogenic strains of *Erwinia herbicola* (26).

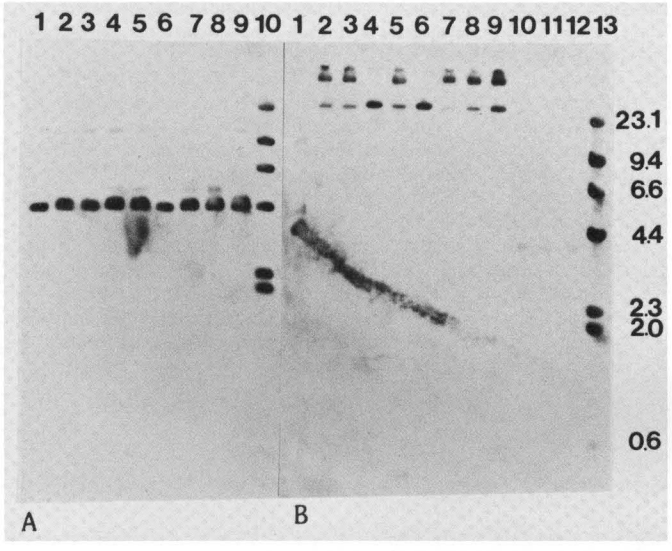

Fig. 3. Southern blots of genomic DNA isolated from strains of *Xanthomonas campestris* pv. *citri* and digested with *Bam*HI. **A,** type A strains Xc62, Xc63, Xc91, Xc92, Xc98, Xc100, Xc101, Xc115, and Xc160 in lanes 1–9. Biotinylated lambda/*Hin*dIII size markers in lane 10. **B,** type A strain Xc62 in lane 1; type B strains Xc64, Xc69, Xc93, Xc94, Xc96, Xc80, and Xc84 in lanes 2–8; type C strains Xc70, CFBP 2905, and CFBP 2906 in lanes 9–11; type D strain Xc90 in lane 12. Biotinylated lambda/*Hin*dIII size markers in lane 13. The probe was a biotinylated 4.2-kb *Bam*HI fragment isolated from the type A strain Xc62.

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