Indigenous Plasmids in *Xanthomonas campestris* pv. *citri*

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ABSTRACT

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Indigenous plasmid DNA was isolated from *Xanthomonas campestris* pv. *citri* by lysis of cells in alkaline-SDS. The plasmids were detected by electrophoresis in horizontal agarose slab gels. At least seven plasmids with apparent molecular masses of approximately 12–53 megadaltons occurred in six laboratory reference strains and 21 clinical strains of diverse host, geographical, and temporal origin. Plasmids with the same or similar electrophoretic mobility in the six reference strains were distributed in five unique plasmid profiles that were associated with different forms of citrus bacterial canker disease (CBCD). However, no specific phenotypic function(s) was associated with any of these plasmids of *X.c.* pv. *citri*. Plasmid profiles may be useful in epidemiological studies of this pathogen, supplementing other methods of bacterial detection and identification. Furthermore, these plasmids may be useful for evaluating relationships between strains of *X.c.* pv. *citri* associated with different forms of CBCD.

Indigenous plasmids occur in all genera of plant pathogenic bacteria (8). Most of the plasmids that occur in phytopathogenic bacteria are cryptic with no known phenotypic function (8). However, major virulence factors are encoded for by plasmid-borne genes in *Agrobacterium tumefaciens, A. rhizogenes, Pseudomonas savastanoi* (7,31), and possibly *Erwinia stewartii* (8,9). The Ti plasmids of strains of *A. tumefaciens* carry genes responsible for host specificity and crown gall tumor morphology (8,23,36). The possible relationships between plasmids and toxin production in pathovars of *P. syringae* and pathogenicity of *Corynebacterium fascians* remain to be conclusively determined. Other factors that may be related to the ecological fitness of phytopathogenic bacteria may also be plasmid-borne (8).

Only limited information about indigenous plasmids in *Xanthomonas* species is available. Single plasmids with molecular masses of 26 and 43 megadaltons (Mdaltons) were reported to occur in *X. campestris* pv. *pruni* (strain 8D51) and *X.c.* pv. *vitians* (strain 068790), respectively (17). Three plasmids with molecular masses of 18, 40, and 72 Mdaltons were reported to occur in one strain of *X.c.* pv. *manihotis* (22). No phenotypic functions have been associated with any of these indigenous plasmids in *Xanthomonas* species. Five different plasmids were observed in seven isolates of *X.c.* pv. *vesicatoria*; however, none of these plasmids was associated with spontaneous mutation from avirulence to virulence (10). In a survey of plasmids among phytopathogenic xanthomonads, no plasmids were detected in *X.c.* pv. *citri, X.c.* pv. *pruni* (strain 13052), or *X.c.* pv. *oryzae* (strains 507 and 604) (22).

Citrus bacterial canker disease (CBCD) is caused by *X.c.* pv. *citri*. Different forms of CBCD are currently recognized (6,11,33,35) and are considered to be caused by specialized strains or pathotypes of the same pathogen. The nature of the pathogenic variability or specialization among variants of *X.c.* pv. *citri* is not understood. It is possible that indigenous plasmids in *X.c.* pv. *citri* may be related to virulence, aggressiveness, or ecological properties of this pathogen.

Routinely applicable and reliable methods for differentiation of strains of *X.c.* pv. *citri* associated with different forms of CBCD are limited. However, at least some of the variants of *X.c.* pv. *citri* associated with different forms of CBCD can be distinguished by their comparative pathogenicity (12,14,19,28,30,34), physiology (12), bacteriophage sensitivity (12,29), and serology (1,2,4,5,25,27).

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Nevertheless, classification of CBCD-associated strains of *X.c.* pv. *citri* may not be as simple as the classification of the disease based on host range, geographical occurrence, and specific pathogen strain.

The objectives of the work reported here were to determine whether plasmids occur in *X.c.* pv. *citri* and to determine the distribution of plasmids in several strains of *X.c.* pv. *citri* associated with different forms of CBCD and of different host, geographical, and temporal origins. No information is currently available about the occurrence of indigenous plasmids in *X.c.* pv. *citri*.

MATERIALS AND METHODS

Bacterial strains. Strains XC59, XC62, XC63, XC64, XC69, and XC70 of *X.c.* pv. *citri* (Table 1) were maintained on Wakimoto's semisynthetic potato medium (5) and stored under sterile mineral oil at 3 C.

An additional 16 strains of *X.c.* pv. *citri* were provided by D. Zagory. Nine of these strains were isolated from orange, grapefruit, lemon, and 'Mexican' lime affected by the Asiatic form of CBCD (CBCD-A) in Argentina, Brazil, Japan, and Paraguay. The remaining seven strains were isolated from cancrosis B (CBCD-B)-affected lemon in Argentina. The Argentine CBCD-B strains were maintained on modified Wilbrink's medium (5 g of peptone, 10 g of sucrose, 0.5 g of K_2HPO_4, and 0.3 $MgSO_4 \cdot 7H_2O$ per liter) (20,24) containing 1.5% Difco purified agar (R. Stall and D. Zagory, *personal communication*).

Five strains of *X.c.* pv. *citri* from Reunion Island were provided by B. Aubert. The form of CBCD with which these five strains were associated was not specifically identified. However, these strains were sensitive to lysis by citriphages CP1 and CP2 but not by citriphage CP3.

Strains SW2 and SS104 of *E. stewartii* were provided by D. L. Coplin and were stored at −20 C in L-broth (10 g of casein hydrolysate, 10 g of NaCl, 5 g of yeast extract, 1 L of distilled water) (17) with 40% glycerol (9).

For plasmid DNA preparation, strains were grown at 28–30 C in 10–20 ml of L-broth, or in modified Wilbrink's medium liquid shake cultures (20,24).

Plasmid DNA isolation. The technique of Kado and Liu (17), with slight modification, was used for plasmid isolation. Cells were collected from L-broth or modified Wilbrink's medium shake cultures by centrifugation at 7,000–10,000 rpm (Beckman JA-20 rotor) for 15 min at 5 C. Each pellet was resuspended in 0.1–1.0 ml of TAE buffer (0.04 M tris, 0.02 M sodium acetate, 0.002 M disodium EDTA adjusted to pH 7.8 with glacial acetic acid). The aqueous phase was usually analyzed directly by agarose gel electrophoresis. Alternatively, the DNA in the aqueous phase was precipitated by the addition of two volumes of cold 95% ethanol and one-tenth volume of 5 M potassium acetate at −20 C. Some DNA preparations were heated at 93–96 C in an attempt to selectively eliminate chromosomal DNA and to resolve plasmid DNA that migrated during electrophoresis in agarose gels to the same position as chromosomal DNA.

Agarose gel electrophoresis. Plasmid DNA preparations were analyzed by electrophoresis in horizontal 0.5, 0.7, and 1.0% SeaKem LE agarose (Marine Colloids, Rockland, ME) slab gels 28 cm long, 14.5 cm wide and 3–4 mm thick. Electrophoresis was carried out at 5V/cm for 5–6 hr (9). The agarose slab was cooled from below with ice water. After electrophoresis, the gels were soaked for 30 min to overnight in 0.5–1 μg of ethidium bromide per milliliter of distilled water. The fluorescence of nucleic acid-containing bands was visualized with a C-63 Transilluminator (Ultraviolet Products, Inc., San Gabriel, CA) fitted with a 302-nm filter. Gels were photographed with a Polaroid MP-4 Land camera using T55 P/N or T665 P/N film through a Wratten No. 9 (yellow) filter.

Molecular masses were calculated by linear regression analyses of log relative mobility in 0.7% agarose versus log molecular mass by using the 16.7–69.8 Mdaltons cryptic plasmids of strains SW2 and SS104 of *Erwinia stewartii* as reference markers (9).

The structural nature of the DNA in bands visualized in the gels was determined by several methods: relative electrophoretic mobilities of the bands were compared in gels of different concentrations (9,16); samples were heated at 93–96 C or in a boiling water bath for 1–5 min and rapidly cooled in ice water (9,16,17) before electrophoresis to selectively remove open circular (OC) and linear (L) DNA; samples were adjusted to 0.1 N NaOH, incubated for 15 min at room temperature and neutralized with an equal volume of 0.1 N HCl (22) to selectively denature OC and L forms of DNA; and samples were adjusted to contain 20 μg of ethidium bromide per milliliter and irradiated under a shortwave (254 nm) UV lamp (C-61, Ultra-Violet Products, Inc., San Gabriel, CA) at a distance of 15 cm for 5–15 min in the dark (15) to selectively nick or partially relax covalently closed circular CCC DNA to OC DNA (9,15).

RESULTS

Plasmid isolation. Preliminary attempts to isolate plasmid DNA from strains XC59, XC62, XC63, XC64, XC69, and XC70 of *X.c.* pv. *citri* by following various protocols (8) were not successful or reproducible. However, plasmid DNA was successfully isolated from these strains by the technique described by Kado and Liu (17) using alkaline (pH 12.6) sodium dodecyl sulfate at elevated temperature. At least seven plasmid-containing bands were readily detected by electrophoresis in horizontal 0.5, 0.7, and 1.0% agarose slab gels (Fig. 1 and Table 2). Resolution of all plasmid DNA bands was best in 1% agarose gels as plasmid DNA with apparent molecular mass of 21–23 Mdaltons migrated to about the same position in these gels as the linear, chromosomal (and/or plasmid) DNA.

Treatment of the cell lysates at 55–65 C for 15–20 min was not always effective for eliminating all linear chromosomal, and possibly linear plasmid, DNA. Heating the plasmid DNA-containing lysates at 93–96 C for 5 min followed by rapid cooling in an ice water bath (17) minimized the amount of linear DNA detected in gels after electrophoresis but resulted in increased

Table 1. *Xanthomonas campestris* pv. *citri* reference strains used in this study of indigenous plasmids

Strain designation[a]				Origin		
Lab	Source	CBCD type[b]	Citriphage type[c]	Host	Country	Source[d]
XC59	IBBF-164	A	$CP1^R,CP2^S,CP3^R$	*Citrus aurantifolia*	Brazil	1
XC62	6501	A	$CP1^S,CP2^R,CP3^R$	*Citrus* sp.	Japan	2
XC63	7801	A	$CP1^R,CP2^S,CP3^R$	*Citrus* sp.	Japan	2
XC64	B-4 (LMLB7)	B	$CP1^R,CP2^R,CP3^S$	*Citrus limon*	Argentina	3
XC69	XC-11	B	$CP1^R,CP2^R,CP3^S$	*Citrus limon*	Argentina	4
XC70	IBSP-512	C	$CP1^R,CP2^R,CP3^R$	*Citrus aurantifolia*	Brazil	4

[a] Strain designations assigned in the Fruit Laboratory, Horticultural Science Institute, Beltsville, MD.
[b] CBCD = citrus bacterial canker disease. CBCD-A strains are associated with the most virulent Asiatic form of CBCD. CBCD-B strains are associated with the cancrosis form of CBCD. CBCD-C strain is associated with the Mexican lime cancrosis in Brazil.
[c] Citriphages CP1 and CP2 were received from M. Koizumi. Citriphage CP3 was received from M. Goto. S = susceptible to lysis at routine test dilution (RTD); R = resistant to lysis at RTD.
[d] 1 = V. Rossetti, Divisao de Patologia Vegetale, Instituto Biologicao, São Paulo, Brazil. 2 = M. Koizumi, Fruit Tree Research Station, Kuchninotsu, Nagasaki, Japan. 3 = M. Goto, Shizuoka University, Shizuoka, Japan. 4 = J. W. Miller, INTA, Bella Vista, Argentina.

diffusion and streaking of fluorescent material (*unpublished*). However, this treatment also resulted in the appearance of additional rapidly migrating bands (*unpublished*).

Plasmid analyses. Five unique plasmid DNA profiles containing one or two prominent ethidium bromide-staining bands were associated with all of the reference strains of *X.c.* pv. *citri* (Fig. 1 and Table 2). The plasmid DNA profiles of strains XC59, XC62, XC63, and XC69 contained one additional band of low-staining intensity. Plasmid DNA preparation of strain XC70 contained three additional low-staining-intensity bands. The electrophoretic mobilities of all of the low-staining-intensity bands were slower than those of the prominently staining bands. The number, relative position, and relative ethidium bromide-staining intensity of all of the bands in 0.5, 0.7, and 1% agarose slab gels were constant and reproducible. The low-staining-intensity bands in the 50–60 Mdalton range (except the 53-Mdalton plasmid band in strain XC63) may be CCC plasmid DNA. However, the possibility that they are artifacts representing structural variants of smaller plasmids cannot be precluded.

The apparent molecular masses of the plasmid DNAs in the six reference strains of *X.c.* pv. *citri* following electrophoresis in 0.7% agarose gels ranged from approximately 12 to 53 Mdaltons. A plasmid with an apparent molecular mass of 21–23 Mdaltons occurred in five of the six strains, and was not detected in strain XC63. Strain XC63 was the only strain that contained a smaller plasmid with an apparent molecular mass of 12 Mdaltons. The unique 53- or 12-Mdalton plasmids in strain XC63 might have been derived from one of the "common" 21- to 23-Mdalton plasmids that occurred in the other strains (9). The plasmid DNA profiles of strains XC59 and XC62 were identical. Strain XC59 was isolated from CBCD-A-affected Key lime in Brazil and is susceptible to lysis by citriphage CP2. Strain XC62 was isolated from CBCD-A-affected *Citrus* sp. in Japan and is susceptible to lysis by citriphage CP1. The plasmid DNA profiles of the CBCD-B strains XC64 and XC69 were nearly identical. The prominent band associated with the plasmid-enriched DNA of strain XC69 had a slightly slower electrophoretic mobility than the corresponding band from strain XC64. Furthermore, the 32-Mdalton plasmid DNA in strain XC69 was not detected in strain XC64.

Based on the following results, each of the major, prominent bands in the six reference strains was identified as CCC DNA. The relative order of electrophoretic migration was the same in 0.5, 0.7, and 1.0% agarose gels. None of the fluorescent bands was eliminated, nor was the electrophoretic migration of any of the bands affected by heating samples at 93–96 C followed by rapid cooling in an ice water bath. These bands were not eliminated by heating samples in a boiling water bath for 1–5 min followed by rapid cooling in an ice water bath; however, the relative intensity of the fluorescence of the ethidium bromide-stained bands was reduced compared to that obtained with unheated samples. All of the low-staining-intensity bands were eliminated when samples were heated in a boiling water bath and rapidly cooled before electrophoresis. The low-staining-intensity 210-Mdalton plasmid DNA from strain SW2 of *E. stewartii* was also eliminated by heating; however, this band reportedly contained CCC plasmid DNA (9). All of the fluorescent bands containing the presumptive CCC plasmid DNAs, but not the band containing linear chromosomal DNA, were eliminated when samples were incubated with 20 μg of ethidium bromide per milliliter and irradiated with UV light before electrophoresis. Most of the DNA in these preparations did not enter the gel. In addition, none of the fluorescent, ethidium bromide-stained bands in the first dimension 1% agarose gel and exposed to UV light migrated into 1% agarose when re-electrophoresed in a second direction (16,32). These results are consistent with nicking or partial relaxation of CCC DNAs to OC forms by ethidium bromide (15). Treatment of plasmid DNA preparations with 0.1 N NaOH for 15 min followed by neutralization did not eliminate any of the bands. Nevertheless, there was increased streaking of ethidium bromide-staining material in the tracks.

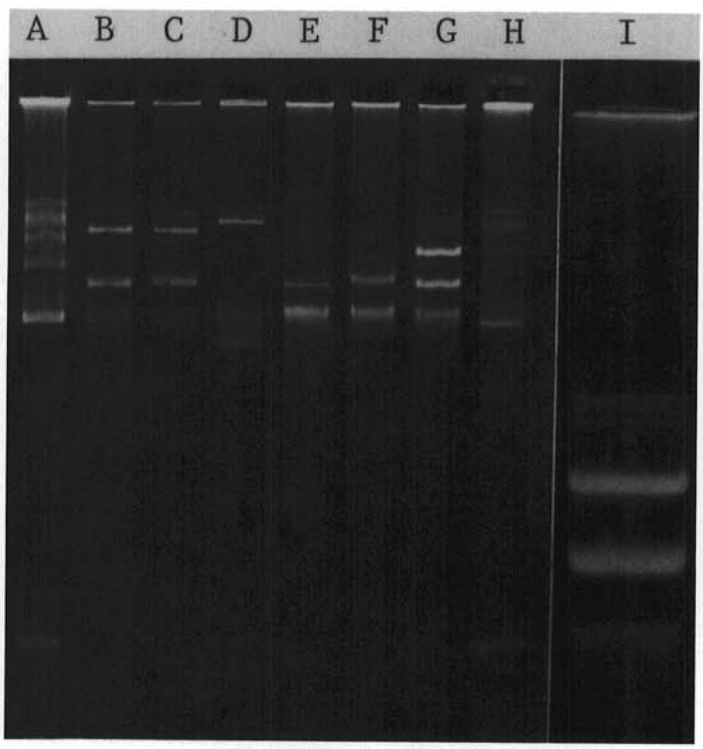

Fig. 1. Agarose (1%) gel electrophoresis of plasmid-enriched DNA from six strains (B through G, I) of *Xanthomonas campestris* pv. *citri* and two strains (A and H) of *Erwinia stewartii*. The *E. stewartii* plasmids from strains SW2 and SS104 were used as standard molecular mass markers. Lane A, SW2; lane B, XC59; lane C, XC62; lane D, XC63; lane E, XC64; lane F, XC69; lane G, XC70; lane H, SS104; and lane I, plasmid-enriched DNA from *X.c.* pv. *citri* strain XC70 as in G and photographically enhanced to show the three slowly migrating plasmid bands that have low-staining intensity with ethidium bromide. The common diffuse band (19–20 Mdaltons) in each lane is presumably linear DNA, mostly of chromosomal origin.

TABLE 2. Plasmid DNAs in six reference strains of *Xanthomonas campestris* pv. *citri*

Strain	Plasmid pattern type	Plasmid molecular mass[a] (Mdaltons)	
XC59	I	45.5 ± 2.2	21.5 ± 0.8
XC62	I	44.8 ± 2.8	21.7 ± 0.6
XC63	II	53.2 ± 0.5	12.3 ± 0.1
XC64	III	21.0 ± 0.3	
XC69	IV	31.7 ± 1.1	23.0 ± 0.4
XC70	V	34.1 ± 0.9	22.3 ± 0.4

[a]Each value represents the mean apparent molecular mass calculated by linear regression analyses of the $\log_{10}$ molecular mass versus $\log_{10}$ relative mobility using the plasmids from *Erwinia stewartii* strains SW2 as molecular mass standards. The relative mobility was determined by measuring the distance in centimeters of each band from the origin on Polaroid T55 film prints. These values were obtained by electrophoresing aliquots of the same preparations in three separate 0.7% agarose gels at 5V/cm for 5.33 hr in tris, sodium acetate, EDTA (TAE) buffer.

TABLE 3. Distribution of plasmid DNAs in 27 strains of *Xanthomonas campestris* pv. *citri*

Plasmid size class (Mdaltons)	Strains (no.)	Percent
53–54	2	7.4
44–46	10	37.0
43–44	2	7.4
34	1	3.7
31–32	8	29.6
20–23	21	77.8
12	1	3.7

The plasmid isolation procedure (12) was designed for gentle extraction of plasmids of various size (2.6–350 Mdaltons). Furthermore, the 13 and 11 plasmids in strains SW2 and SS104 of *E. stewartii*, respectively (9), were resolved by this procedure in the present study. No additional bands containing putative OC or linear forms of any of these plasmid DNAs were detected by electrophoresis of any plasmid DNA preparations from these strains.

Plasmids in other strains. The alkaline SDS procedure (17) was used to screen for plasmids in nine additional CBCD-A strains of *X.c.* pv. *citri*; seven CBCD-B strains; and five strains isolated from CBCD-affected *Citrus* spp. in Reunion Island. All of these strains contained plasmids. The apparent molecular masses of these plasmids ranged from about 20 to 54 Mdaltons. The plasmid profiles of the nine CBCD-A-associated strains and the five strains from Reunion Island were generally similar to those of the reference strains XC59, XC62, and XC63. The plasmid DNA from one of the strains (JA162.5) from Reunion Island migrated as a single broad diffuse band in 1% agarose. Similarly, the plasmid DNAs detected in the seven CBCD-B-associated strains migrated as two bands with electrophoretic mobilities similar to the 21- to 23- and 31- to 32-Mdalton plasmids in the reference strains XC64 and XC69. A plasmid with an electrophoretic mobility of the 12-Mdalton plasmid that occurred in the reference strain XC63 was not detected in any of these additional 21 strains. None of the plasmid profiles of these 21 strains was similar to that of reference strain XC70. The plasmid number and apparent size classes based on the electrophoretic mobility in these strains were similar to those in the reference strain of *X.c.* pv. *citri*; however, the precise molecular masses of these plasmids need to be determined.

The distribution of seven plasmids in these strains of *X.c.* pv. *citri* of diverse origins is presented in Table 3. The plasmid band migrating with an apparent molecular mass of 20–23 Mdaltons was the most frequently occurring plasmid among these strains. However, the range of plasmid sizes and profiles may be larger than that based on the strains used in this study.

DISCUSSION

This is the first report of the occurrence of indigenous plasmids in *X.c.* pv. *citri*, and is the first evaluation of the comparative distribution of plasmids in pathogenic variants of a pathovar of *X. campestris*. At least seven different plasmids occurred in the six reference strains and in an additional 21 strains of *X.c.* pv. *citri* of diverse host plant, geographical, and temporal origin examined in this study. Nevertheless, the existence of larger and/or labile plasmids in *X.c.* pv. *citri*, not detected by the plasmid isolation and detection technique (17), cannot be precluded.

The structural nature of the minor, low-staining-intensity bands was not conclusively determined. They may represent plasmids that are present in low copy numbers. Alternatively, they may be extraction artifacts representing other forms of the smaller plasmids with lower superhelicity. The possibility that they represent rearrangements of the smaller plasmids cannot be precluded. Finally, the efficiency with which these plasmids are extracted by this method may be low.

The only xanthomonad in which multiple indigenous CCC plasmid DNAs have been reported is *X.c.* pv. *manihotis* (22). This was based in part on the apparent stability of three distinct CCC plasmid DNAs treated with alkali at pH 12.2 for 15 min followed by neutralization with HCl.

As no functions or phenotypes are known for any of these plasmids of *X.c.* pv. *citri*, they are considered to be genetically cryptic. The possible relationship(s) of any of these plasmids to virulence and/or ecological fitness of strains of *X.c.* pv. *citri* remains to be determined. However, with respect to epidemiology, the plasmid profiles of the strains of *X.c.* pv. *citri* associated with different forms of CBCD were unique. Furthermore, strains associated with CBCD-A contain major plasmids with apparent molecular masses of 43–44, 45–46, or 53 Mdaltons. In contrast, the less aggressive strains with restricted host range, and associated with CBCD-B, contain major plasmids of 21–23 and 31–32 Mdaltons. In addition, one CBCD-C strain contained a 31–32 Mdalton plasmid. Thus, the differentiation of these strains of *X.c.* pv. *citri* based on plasmid content is consistent with differentiation of these strains by serology (1,2,4,5,12,25–27) and by phage sensitivity (12,29).

The plasmids in 15 strains of *X.c.* pv. *citri* from Reunion Island were isolated and analyzed by techniques other than those used here (E. Laville, *personal communication*). Based on the number of distinct ethidium bromide-staining bands visible after electrophoresis, these strains contained 8–10 presumptive plasmids. However, the structural nature of the material in the electrophoretic bands was not determined. Each of these 15 strains was reported to contain one (12 strains) or two (three strains) plasmids with apparent molecular weights of 4–46 Mdaltons.

Plasmid profiles, therefore, may provide bacterial fingerprints useful for identifying isolated strains in epidemiological studies as has been reported for other bacteria (3,34). Moreover, specific plasmid DNA sequences of *X.c.* pv. *citri* in lesions in planta or in colonies on filters could potentially be detected by hybridization techniques (13) by utilizing specific plasmid DNA probes. Sensitive detection, as well as rapid and precise identification of *X.c.* pv. *citri* in presumptive CBCD lesions, could be made even in the presence of other bacteria.

The molecular relatedness among these plasmids remains to be determined. Comparative digestions of these plasmids by restriction endonucleases, molecular hybridizations, and more precise size and structure determinations, should resolve molecular identity and/or diversity among these plasmids.

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