Isozyme Analysis of *Xanthomonas campestris* pv. *citri*

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ABSTRACT

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Isozyme analysis of 14 putative isozymic loci by horizontal starch gel electrophoresis was conducted on 36 strains of *Xanthomonas campestris* pv. *citri* representing four pathogenic variants associated with different forms of citrus bacterial canker disease in eight countries. An additional 80 strains of *X. campestris* associated with citrus bacterial spot disease, primarily in Florida citrus nurseries, also were analyzed. Four enzymes were monomorphic in all 116 strains. The number of isomorphs for the 10 remaining polymorphic loci ranged from two to five. Generally, all strains of *X. c. citri* were isozymically similar, but not identical in all cases, to the neopathotype strain. No isozymes were found in the citrus canker groups of strains that distinguished any of the forms of citrus canker. As a subgroup, the Asiatic citrus canker strains exhibited relatively little isozymic polymorphism despite their varied origins worldwide. In contrast, several isozymic alleles were present only in the set of citrus bacterial spot strains isolated from Florida citrus nurseries. These strains also exhibited extensive isozymic polymorphism. Isozyme analysis may be a useful technique in epidemiological studies of phytopathogenic bacteria.

Citrus bacterial canker disease (citrus canker) is caused by *Xanthomonas campestris* pv. *citri* (Hasse) Dye. Different forms of citrus canker are considered to be caused by pathogenic variants of the same pathogen distinguished by host range, geographical distribution, pathogenicity, serology, phage typing, plasmid DNA analysis, and genomic fingerprinting (4–6,11). At least three groups of strains of *X. c. citri* are recognized (4). Group A strains are associated with Asiatic citrus canker, group B strains with cancrosis B in Argentina and Uruguay, and group C strains with Mexican lime cancrosis in Brazil. Presumptive strains of *X. c. citri* are associated with citrus bacteriosis in Mexico (4). Strains in each of these groups produce raised lesions. A genomically heterogeneous group of strains of *X. campestris* (11) is associated with a citrus bacterial spot (citrus spot) disease, primarily in nurseries, in Florida. These strains generally produce flat, water-soaked, or slightly raised lesions with associated necrosis and chlorosis (18). The relationship(s) of these strains to variants of *X. c. citri* is not understood.

Isozyme electrophoresis is widely used to assess genetic relatedness and variation within and among populations of bacteria (3,9,15,19,20, Bonde et al, *unpublished*). Isozyme analysis is also a complementary tool for determining taxonomic relationships in eukaryotes (1,2,8,13,14). The applicability of isozyme analysis is dependent on the extent of existing enzyme variation. Because the enzymes assayed are constitutively expressed, many genetic loci may be studied regardless of environmental conditions under which the strains were grown. Comparisons of isozyme patterns are possible. These can be used in conjunction with other genetic traits to determine taxonomic relationships among phytopathogenic bacterial strains. Differential isozyme patterns are related to distinct pathovars of *X. campestris* (Bonde et al, *unpublished*).

The purpose of this study was to examine the extent of isozyme variation within different pathogenic variants and in widely separated populations of *X. c. citri*, and the xanthomonads associated with citrus bacteriosis in Mexico and citrus spot in Florida.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All strains were maintained in the U.S. Department of Agriculture collection of phytopathogenic bacteria in Beltsville, MD. A total of 116 strains from Argentina, Brazil, Florida, Guam, Japan, Mexico, North Yemen, and Pakistan were examined. They are maintained aseptically on Wakimoto semi-synthetic potato medium under sterile mineral oil at 3 C (5,6). The strains represented a wide range of types based on a diversity of pathogenic variants, geographical origin, length of time in culture, and hosts from which they were isolated. The purity, pathogenicity, and phage sensitivity of each strain were verified before isozyme analysis. The lysate from each strain was analyzed at least twice.

Culture conditions and preparation of bacterial enzyme lysates. Each strain was grown from a single colony in 100 ml of M9 broth (12) supplemented with 0.2% (w/v) Difco casamino acids and 0.1% (w/v) Difco yeast extract at 30 C on an orbital shaker (120 rpm) for 24 hr. The initial pH of the medium was 6.8. Cells were harvested by centrifugation at approximately 5,500 *g* for 10 min at 4 C, resuspended once in 100 ml of ice-cold sterile saline (0.9% NaCl) plus 0.1% (v/v) Triton X-100, and washed in ice-cold sterile deionized water. After the final wash, the supernatant was quickly decanted and the inside of the tube was wiped dry. The cell pellet was resuspended in the remnant liquid and transferred to a sterile 1.5-ml Eppendorf microcentrifuge tube. The tubes were stored at −60 C until needed. Before electrophoresis, the frozen bacterial suspension was thawed on ice, frozen by submersion in liquid nitrogen, and macerated with a glass rod or a Teflon pestle previously dipped in liquid nitrogen. The pellet then was thawed in ice before refreezing in liquid nitrogen. These steps were repeated once. After the final thawing, two or three drops of ice-cold 50 mM Tris-HCl buffer, pH 7.1, were added to the pellet. The tube was centrifuged at full speed in a microcentrifuge for 3 min. The lysate was drawn off in wicks of Whatman 3MM filter paper and subjected to electrophoresis (40 wicks/gel). The remnant lysate was stored in the same tube.

Electrophoresis. Horizontal starch gel electrophoresis was performed using 14% (w/v) hydrolyzed starch (Sigma Chemical Co., St. Louis, MO) as previously described (10). Staining techniques were those of Micales et al (14). The enzymes assayed

and the abbreviations used in the text are listed in Table 2. During the course of this study, other enzymes were resolved, although they could not be scored consistently. We report here only those routinely assayed enzymes that produced consistent results.

Genetic nomenclature. Isomorphs at a particular locus were determined by the anodal migration distance (mm) of the enzymes from the origin. The isozyme band with the slowest migration rate was designated as "a" and isozyme bands with increasing anodal migration rates were alphabetized sequentially.

RESULTS

Selected bacterial strains were grown in five culture media to determine if nutrients available during culturing affected the isozyme variation of *X. c. citri* and other xanthomonads isolated from citrus. Although cell yield was variable (data not shown), culture medium had no effect on enzyme migration. Thus, each enzyme system in this study was constitutively expressed rather than inducibly expressed.

There was a single major band of activity for each enzyme. The staining intensities of the isozyme bands were not compared because the presence or absence of the specific allozymes was determined. Each band for each enzyme migrated anodally from the origin. Ill-defined zones of activity and secondary minor bands occurring anodally or cathodally to the major band were associated with some strains. These appeared to have no consistent pattern and probably were due to binding of the enzyme to faster and/or slower migrating contaminants, conformational changes in the molecule, complexes with cofactors or other ions, or even incompletely transcribed or translated products (2). No null alleles at any locus were detected.

The number of presumptive isozymic loci and the number of isomorphs per locus were determined from the gel patterns. Four of the 14 enzymes assayed, GDH, LDH, NP, and SOD, were

TABLE 1. Bacterial strains used for isozyme analysis

Strain				
Name	Laboratory designation	No.	Geographical origin	Remarks[a]
Xanthomonas campestris pv. *citri*	XC115	1	New Zealand	Neopathotype strain (PDDCC-24-85); CBCD-A
	XC59	1	Brazil	CBCD-A
	XC62, XC63	2	Japan	CBCD-A
	XC91, XC92[b]	2	Argentina	CBCD-A
	XC101, XC102	2	Guam	CBCD-A
	XC99, XC100	2	Pakistan	CBCD-A
	XC98	1	North Yemen	CBCD-A
	F132–F134, F196–F200, F202	9	Florida	CBCD-A
	XC64, XC69, XC93,[b] XC94,[b] XC95[b]	5	Argentina	CBCD-B
	XC70	1	Brazil	CBCD-C
	XC90	1	Mexico	CB
X. campestris	G20–G23,[c] T20–T24[d]	9	Mexico	Weak or non-pathogenic strains isolated from CB-affected citrus in Mexico
X. campestris	F1–F6, F12–F15, F18, F19, F25–F30, F33–F37, F42–F44, F47–F59, F61, F62, F67–F69, F72–F81, F83–F89, F93–F101, F105, F108, F114, F116, F117, F120–F123	80	United States (Florida)	CBSD
Total		116		

[a] CBCD-A = Asiatic citrus bacterial canker disease; CBCD-B = cancrosis B form of CBCD; CBCD-C = cancrosis C form of CBCD; CB = citrus bacteriosis; CBSD = citrus bacterial spot disease.
[b] Strains XC91–XC95 were received from D. Zagory, University of Florida, Gainesville, IFAS.
[c] Strains G20–G23 were received from J. Garza L., INTA, Tecoman, Colima, Mexico.
[d] Strains T20–T24 were received from S. Rodriguez, INTA, Tecoman, Colima, Mexico.

TABLE 2. Distribution of isozyme alleles at 14 loci among xanthomonads associated with citrus bacterial canker disease (CBCD-A, -B, -C), citrus bacteriosis (CB), and citrus bacterial spot disease (CBSD)

		Electromorph frequencies of strains associated with:[c]				
Locus[a]	Electromorph[b]	CBCD-A (20)	CBCD-B (5)	CBCD-C (1)	CB (10)	CBSD (80)
AK	a	0.05	0.00	0.00	0.00	0.44
	b*	0.75	0.80	0.00	1.00	0.38
	c	0.20	0.20	1.00	0.00	0.18
FDP	a	0.25	0.80	0.00	0.00	0.78
	b*	0.75	0.20	1.00	1.00	0.22
GDH	a*	1.00	1.00	1.00	1.00	1.00
G6PDH	a	0.00	0.00	0.00	0.00	0.11
	b*	1.00	1.00	1.00	1.00	0.89
GK	a	0.00	0.00	0.00	0.00	0.03
	b	0.15	0.00	0.00	0.00	0.48
	c*	0.85	1.00	1.00	1.00	0.49
GPI	a	0.00	0.00	0.00	0.00	0.07
	b	0.00	0.80	0.00	0.00	0.10
	c	0.25	0.20	0.00	0.00	0.53
	d*	0.75	0.00	0.00	1.00	0.30
IDH	a	0.00	0.00	0.00	0.00	0.34
	b	0.00	0.00	0.00	0.00	0.08
	c	0.00	0.00	0.00	0.00	0.05
	d*	1.00	1.00	1.00	1.00	0.53
LDH	a*	1.00	1.00	1.00	1.00	1.00
MDH	a	0.00	0.00	0.00	0.00	0.02
	b*	1.00	1.00	1.0	1.00	0.96
	c	0.00	0.00	0.00	0.00	0.02
MPI	a	0.15	0.16	0.00	0.00	0.41
	b	0.10	0.00	0.00	0.00	0.19
	c*	0.75	0.83	1.00	1.00	0.40
NP	a	1.00	1.00	1.00	1.00	1.00
PGD	a	0.10	0.00	0.00	0.00	0.20
	b	0.00	0.00	0.00	0.00	0.10
	c	0.05	1.00	0.00	0.00	0.10
	d	0.00	0.00	0.00	0.00	0.10
	e*	0.85	0.00	1.00	1.00	0.50
PGM	a	0.00	0.00	0.00	0.00	0.11
	b	0.00	0.00	0.00	0.00	0.59
	c*	1.00	1.00	1.00	1.00	0.30
SOD	a	1.00	1.00	1.00	1.00	1.00

[a] AK = adenylate kinase; FDP = fructose diphospatase; GDH = glutamic dehydrogenase; G6PDH = glucose-6-phosphate dehydrogenase; GK = glucokinase; GPI = glucose phosphate isomerase; IDH = isocitrate dehydrogenase; LDH = lactate dehydrogenase; MDH = malate dehydrogenase; MPI = mannose phosphate isomerase; NP = purine nucleoside phospherylase; PGD = phosphogluconate dehydrogenase; PGM = phosphoglucomutase; SOD = superoxide dismutase. GDH, LDH, NP, and SOD are nonomorystric enzymes; all others are polymorphic. The continuous buffer system described by Ridgway et al (16) was used for LDH and NP. The discontinuous buffer system described by Clayton and Tretiak (7) was used for all the other isozymes.
[b] The electromorphs with the slowest anodal migration rate are designated as a. Electromorphs with increasing anodal migration rates are designated sequentially as b, c, d, and e. The asterisk indicates electromorphs in neopathotype strain XC115 of *Xanthomonas campestris* pv. *citri*.
[c] The numbers in parentheses indicate total number of strains.

TABLE 3. Distribution of electrophoretically distinct isozymes (isomorphs) at 14 loci in xanthomonads associated with citrus bacterial canker disease (CBCD), citrus bacteriosis (CB), and citrus bacterial spot disease (CBSD) in different geographic areas[a]

Locus[b]	Neopathotype strain	CBCD-A							CBCD-B	CBCD-C	CB	CBSD
		ARG	BRZ	FLA	GUAM	JPN	NYEM	PAK	ARG	BRZ	MEX	FLA
AK	b[c]	b	c	a, b, c	b	b	b	b	b, c	c	b	a, b,* c
FDP	b	a	b	a, b	b	b	b	b	a, b	b	b	a, b*
GDH	a	a	a	a	a	a	a	a	a	a	a	a*
G6-PDH	b	b	b	b	b	b	b	b	b	b	b	a, b*
GK	c	c	c	c	c	c	c	c	c	c	c	a, b, c*
GPI	d	d	d	c, d	d	d	d	d	b, c	c	d	a, b, c, d*
IDH	d	d	d	d	d	d	d	d	d	d	d	a, b, c, d*
LDH	a	a	a	a	a	a	a	a	a	a	a	a*
MDH	b	b	b	b	b	b	b	b	b	b	b	a, b,* c
MPI	c	c	c	a, b, c	c	c	c	c	c	c	c	a, b, c*
NP	a	a	a	a	a	a	a	a	a	a	a	a*
PGD	e	e	e	a, e	e	e	e	e	c	d	d	a, b, c, d, e*
PGM	c	c	c	c	c	c	c	c	c	c	c	a, b, c*
SOD	a	a	a	a	a	a	a	a	a	a	a	a*

[a] ARG = Argentina; BRZ = Brazil; FLA = Florida; JPN = Japan; NYEM = North Yemen; PAK = Pakistan; MEX = Mexico.
[b] AK = adenylate kinase; FDP = fructose diphospatase; GDH = glutamic dehydrogenase; G6PDH = glucose-6-phosphate dehydrogenase; GK = glucokinase; GPI = glucose phosphate isomerase; IDH = isocitrate dehydrogenase; LDH = lactate dehydrogenase; MDH = malate dehydrogenase; MPI = mannose phosphate isomerase; NP = purine nucleoside phospherylase; PGD = phosphogluconate dehydrogenase; PGM = phosphoglucomutase; SOD = superoxide dismutase. GDH, LDH, NP, and SOD are nonomorystric enzymes; all others are polymorphic. The continuous buffer system described by Ridgway et al (16) was used for LDH and NP. The discontinuous buffer system described by Clayton and Tretiak (7) was used for all the other isozymes.
[c] The isozyme with the slowest anodal migration rate is designated as a. Isozymes with increasing anodal migration rates are designated sequentially as b, c, d, and e. The asterisk indicates isomorph in neopathotype strain XC115 of *Xanthomonas campestris* pv. *citri*.

TABLE 4. Coefficients of similarity among the xanthomonad strains used for isozyme analysis

Strains	Group[a]	Rodgers' CS[b]	Standard deviation
Within:	CBCD-A	0.8523	0.1335
	CBCD-B	0.9143	0.0452
	CBCD-C	0.0000[c]	0.0000
	CB	1.0000	0.0000
	CBSD	0.6788	0.1548
Between:	CBCD-A and CBCD-B	0.7686	0.0712
	CBCD-A and CBCD-C	0.8286	0.0848
	CBCD-B and CBCD-C	0.7571	0.0391
	CBCD-A and CBSD	0.6266	0.1144
	CBCD-B and CBSD	0.5835	0.0787
	CBCD-C and CBSD	0.6536	0.1357
	CB and CBSD	0.6357	0.1239
	CBCD-A and CB	0.9143	0.1190
	CBCD-B and CB	0.7871	0.0457
	CBCD-C and CB	0.8571	0.0007

[a] CBCD-A = Asiatic citrus bacterial canker disease; CBCD-B = cancrosis B form of CBCD; CBCD-C = cancrosis C form of CBCD; CB = citrus bacteriosis; CBSD = citrus bacterial spot disease.
[b] Rodgers' coefficient of similarity (CS) as determined by R. E. Straus, 1979, Allozyme. Version 6.0 in FORTRAN invoicing the International Mathematical and Statistical Library.
[c] Only one strain available.

monomorphic (Table 2). The number of isomorphs for each of the remaining 10 polymorphic loci ranged from two to five (Table 2). The mean number of isomorphs per locus was 2.6. No enzyme class was markedly more or less variable than any other.

The isozyme patterns of the 20 Asiatic citrus canker and 10 citrus bacteriosis strains were generally less variable than those of the five cancrosis-B strains (Tables 2 and 3). Only one cancrosis C strain was available for this study. Each isomorph for each locus was represented in the group of Florida citrus spot strains (Tables 2 and 3). Some allozymes (e.g., G6PDHa, GKa, and GPIa) were rare and unique to these Florida citrus strains. The Florida citrus spot strains, as a group, had the greatest degree of isozymic variability; however, more strains of this group were represented than strains in the other groups. The Asiatic citrus canker strains from Guam, Japan, Mexico, North Yemen, and Pakistan were electrophoretically indistinguishable and invariable (Table 3). Among the nine Asiatic citrus canker strains from Florida, five isozymic loci were variable. Asiatic citrus canker strains from Argentina had no variable loci, whereas the cancrosis B strains from Argentina had three variable loci. A few allozymes, such as GDH and G6PDH, were universally distributed among the Asiatic citrus canker, cancrosis B, and Mexican lime cancrosis groups.

The isomorph frequencies are presented in Table 2. The type strain (XC115) isomorph of *X. c. citri* was generally the most common isomorph for each enzyme; however, the highest frequencies of isomorphs in the Florida citrus spot strains were different at the FDP and GPI loci from those in the citrus canker and citrus bacteriosis strains (Table 2). At the PGM locus, an isozyme unique to the citrus spot strains from Florida was the most frequently occurring isozyme in this group of strains. All other isozymes unique to Florida citrus spot strains existed in low frequencies. The individual multilocus combinations of rare and unique isozymes in the Florida citrus spot strains were distinctive for this group of strains. No other group of strains of *X. c. citri* from any other geographical region had any isozyme exclusive to it.

The coefficients of similarity (CS) (17) among the xanthomonad strains in each group and those between groups of strains are given in Table 4. As a group, the Florida citrus bacterial spot disease strains were more heterogeneous (CS = 0.68) than any of the other groups of strains (CS values ranged from 0.85 to 1.00). Generally, the Florida citrus bacterial spot disease strains were not closely related to the Asiatic citrus canker, cancrosis B, Mexican lime cancrosis, and citrus bacteriosis groups of strains (CS values ranged from about 0.58 to 0.64). The CS between the Asiatic citrus canker and citrus bacteriosis groups of strains was about 0.91. This is somewhat higher than the CS between the citrus bacteriosis and either the cancrosis B strains (CS = 0.79) or the single cancrosis C strain (CS = 0.86).

DISCUSSION

Multilocus enzyme electrophoresis was used to assess isozyme variation at structural genes encoding 14 enzymes in 116 strains of *X. c. citri* and other xanthomonads associated with citrus leaf-, twig-, and fruit-spotting diseases from around the world. These enzymes were determined to be constitutively expressed rather than inducibly expressed because the bacterial lysates yielded similar enzyme-active extracts irrespective of the medium used. There were no detectable isozymic phenotypic effects caused by the growth medium. The isozymic loci examined in this study were

assumed to be representative of the genome of *X. c. citri*. Ten loci were polymorphic and four were monomorphic.

The strains used in this study represent a wide range of types with respect to geographic origin, time of isolation, virulence, host species from which isolated, and length of time in culture. However, in the case of the Asiatic citrus canker strains, the recovery of strains having the same electrophoretic genotype in several geographic regions and isolated in different years suggests a low degree of genetic variability and is consistent with a clonal population structure for this pathogenic variant (20). The electrophoretic genotype in all Asiatic citrus canker strains from Argentina, Brazil, Florida, Guam, Japan, North Yemen, and Pakistan is shared with the neopathotype strain of *X. c. citri*. This genotype perhaps was distributed worldwide by dissemination of infected plant material.

The Florida citrus bacterial spot strains of *X. campestris* are a polytypic assemblage of strains. The extensive isozymic variation in the citrus bacterial spot-associated strains is not consistent with the recent introduction of a single strain into Florida. Novel alleles, such as AKa, GKb, MPIa, and PGMb, in the group of Florida strains did not occur in other strains of *X. c. citri*. These novel alleles may be useful markers for identification of these strains. The number and diversity of isozymic variants in the group of Florida citrus bacterial spot strains corroborates the pathogenic, serological, plasmid DNA, and genomic fingerprint variation in this group of strains (11,18).

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