Differentiation of Genomic Structure by rep-PCR Fingerprinting to Rapidly Classify *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

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DNA primers corresponding to repetitive extragenic sequences (repetitive extragenic palindromic [REP], enterobacterial repetitive intergenic consensus [ERIC], and BOX element [BOX1A] sequences) and polymerase chain reaction (rep-PCR) were used to generate complex fingerprint patterns that identified four distinct genotypes among strains classified as *Xanthomonas campestris* pv. *vesicatoria*. After agarose gel electrophoresis, these genotypes were easily differentiated from each other by comparing the migration rates of 60 or more bands generated with rep-PCR. Representative strains of each genotype were pathogenic to tomato and/or pepper. We performed rep-PCR on numerous strains that have been included in previous studies, and our observations using the simple, rapid procedure of rep-PCR were consistent with the polyphasic approaches published by others. The majority of strains belonged to two previously described groups, A and B. Group A strains originated from tomato or pepper. Most of these strains proved to be negative in starch hydrolysis and pectolytic activity tests. All group A strains were relatively homogeneous with regard to their rep-PCR fingerprint patterns. Group B strains originated primarily from tomato and were positive for starch hydrolysis and pectolytic activity. Numerous rep-PCR fingerprint polymorphisms distinguished six patterns or lineages in group B. Group B strains comprised an important component of the tomato spot complex in the Northcentral tomato production region of North America. Three strains comprised two additional genotypes and were clear outliers compared to strains classified as group A or B. Interestingly, based on rep-PCR genomic fingerprint patterns, two of the nongroup A/B strains shared numerous bands of similar mobility with strains pathogenic for cabbage, classified as *X. c.* pv. *campestris*, suggesting that these two solanaceous strains are closely related to the cabbage pathogen.

Additional keywords: bacterial spot, genetic diversity, integrated disease management, population structure, strain identification.

Xanthomonas campestris pv. *vesicatoria*, the causal agent of bacterial spot on pepper and tomato, was first diagnosed in the early 1920s (7,9,16). *X. c. vesicatoria* occurs worldwide in regions of pepper and tomato production (15,32). On tomato, *X. c. vesicatoria* affects all above ground plant tissue and can incite marketable yield losses ranging from 5 to 70% (28,32). Routine application of bactericides, such as copper or streptomycin, do not provide consistent control, because of low efficacy (14) and the ability of populations to acquire resistance to the bactericides (26,35). Cultural practices, such as burial of crop debris, crop rotation, and use of windbreaks to limit on-farm incidence of spot in tomato, have been recommended (19,32) and implemented. However, farm-level integrated disease management practices appear to have a minimal impact on disease control, especially when weather conditions favor the spread of the pathogen. Ultimately, disease control is likely to be achieved primarily through disease management strategies implemented before the seeds (or transplants) arrive at the farm, such as the development of resistant cultivars and implementation of protocols designed to limit the introduction of initial inoculum (13).

Breeding for durable disease resistance and implementing necessary detection/diagnostic protocols have posed a challenge because *X. c. vesicatoria* is phenotypically, serologically, pathogenically, and genotypically diverse (2–4,8,20,22,27,34,36,37,40). For example, genetic resistance was developed in tomato (30), but virulent isolates were identified (40) before the resistant line was commercially deployed. An understanding of the genetic diversity of strains that comprise the population of *X. c. vesicatoria* should prove useful for devising disease management strategies.

Assessment of diversity based on fatty acid profile analysis, DNA homology studies, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins enabled Vauterin et al (36,37) to group *X. c. vesicatoria* strains into two subpathovar categories, subgroups A and B. Likewise, Jones et al (20), Stall et al (34), and Bouzar et al (3) have classified *X. c. vesicatoria* strains at the subpathovar level as groups A and B based on serology, DNA hybridization studies, DNA restriction enzyme digestion profiles, protein profile analysis, and other techniques.

Recently (1990–1994), numerous epidemics of bacterial spot have occurred in commercial greenhouses (on tomato transplants) and in commercial fields throughout the Northcentral production region of North America (Michigan, Ohio, and Indiana and Ontario, Canada). Using a genomic DNA fingerprinting protocol, we

initiated a study to determine the genetic diversity of these strains compared to strains from other parts of the world. This rapid and highly reproducible method employs primers corresponding to repetitive extragenic sequences (repetitive extragenic palindromic [REP] [10,17], enterobacterial repetitive intergenic consensus [ERIC] [18,31], and BOX element [BOX1A] [25] sequences) to generate complex fingerprint patterns from DNA of bacteria in combination with the polymerase chain reaction (PCR) protocol (rep-PCR; 6,38,39).

We previously reported that rep-PCR effectively differentiated two genotypes, or evolutionary lines, among strains classified as *X. c. vesicatoria* based on overall chromosomal organization (24) and that these two genotypes conform to the divisions described by others (34,37). In our current study, we used rep-PCR to extend this analysis by determining the relative genetic diversity among strains within groups A and B and by identifying two additional genotypes able to incite disease on pepper or tomato. Genotypes, as defined by rep-PCR, correlated with selected phenotypic characteristics, such as amylolytic and pectolytic activity, highlighting the utility of rep-PCR in distinguishing phytopathogenic bacteria at the pathovar and subpathovar levels. This is also the first study to report that amylolytic/pectolytic strains (subgroup B strains, *sensu* Vauterin et al [37], and group B or T2 strains, *sensu* Stall et al [34]) constitute an important component of the tomato spot complex in the Northcentral tomato production region of North America.

MATERIALS AND METHODS

Bacterial strains, isolation of chromosomal DNA, and PCR conditions. Original strain designation(s), geographic origin, year of isolation, race designations, and sources of bacterial strains or genomic DNA are listed in Table 1.

Maintenance and culture of bacteria, preparation of total genomic DNA, PCR conditions, and electrophoresis conditions were as described previously (24), except PCR amplification was performed in a model 110s Tempcycler II (Coy Corporation, Grass Lake, MI) or a Perkin-Elmer thermocycler (Perkin-Elmer, Norwalk, CT). The protocols using REP, ERIC, and BOX primers are referred to as REP-PCR, ERIC-PCR, and BOX-PCR, respectively, and rep-PCR collectively (39). Each PCR experiment included a control lacking template DNA. Differences in fingerprint patterns between genotypes were assessed visually.

Similarity coefficients among 57 group A strains and 23 group B strains were determined using the program similarity for qualitative data (SIMQUAL), NTSYS-PC (29). The total number of unique scorable bands for all strains within each group was determined and bands were scored as present (1) or absent (0) for each strain. Clustering analysis was performed by the unweighted pair-group method and dendrograms generated using the SAHN subroutine of NTSYS-PC. Clustering analysis was performed on data generated from each primer set separately and on the combined data sets generated from the three different primers. Analysis of combined data is particularly useful for determining genetic relationships (21,23).

Physiological and biochemical characterization. All strains were evaluated for amylolytic and pectolytic activity (3,5) and for cellulolytic activity (11), which are assays commonly used to characterize *X. c. vesicatoria* strains (12). Colony characteristics on a basal CKTM medium lacking antibiotics were recorded as described by Sijam et al (33). All physiological and biochemical tests were repeated a minimum of two times for each strain.

Pathogenicity tests and race determination. Pathogenicity and race determination of many strains used in this study have recently been reported by Stall et al (34) and Bouzar et al (2,3). Pathogenicity of other selected strains were evaluated with tomato cultivar Bonny Best and accession Hawaii 7998. Plants were pretreated by enclosing them in large plastic bags (i.e., 100% relative humidity) for 24 h after thorough misting with water. Plants were briefly uncovered, misted with a bacterial suspension (1×10^8 cfu/ml), and maintained in plastic bags for an additional 24–36 h. The plastic was removed, and plant symptoms were recorded up to 21 days postinoculation. Race determination also was assessed as described previously (20,27).

RESULTS

Four genotypes resolved by REP-, BOX-, and ERIC-PCR. DNA fingerprints were generated from total chromosomal DNA extracted from 83 strains of *X. c. vesicatoria* originating from various parts of the world (Table 1). Primers corresponding to REP, BOX, and ERIC sequences, in combination with PCR, generated complex genomic fingerprinting patterns from DNA of each strain consisting of 20 or more PCR products that ranged in size from approximately 0.2 to more than 5 kb. Strains were classified into four distinct genotypes based on these fingerprint patterns (Fig. 1). The REP- (Fig. 1, lanes 1–6), BOX- (Fig. 1, lanes 7–12), and ERIC-PCR (Fig. 1, lanes 13–18) experiments were equally effective in delineating the four genotypes. The majority of strains belonged to group A (*sensu* 35,38) (Fig. 1, lanes 1, 7, and 13) or group B (Fig. 1, lanes 2, 8, and 14) representing 57 (69%) and 23 (28%) of the strains analyzed, respectively. The third genotype comprised a single strain, Xv441 (Fig. 1, lanes 3, 9, and 15), and the fourth genotype included two strains, DC91-1 and DC92-6 (Fig. 1, lanes 4 and 5, 10 and 11, and 16 and 17). No bands common to all genotypes were noticeably generated by the REP-, BOX-, and ERIC-PCR experiments. In the ERIC-PCR experiment, one to three bands appeared to comigrate among strains classified as group A or B. For example, strain ATCC 11633 (Fig. 1, lane 13) yielded three bands (highlighted by arrowheads in lane 13) that comigrated with bands generated from chromosomal DNA of Xcv736 (Fig. 1, lane 14). Sequencing or hybridization studies would need to be conducted to determine if the comigrating bands are analogous portions of DNA in both the group A and B strains.

Based on total chromosomal fingerprint patterns, the rep-PCR experiments effectively differentiated four genotypes among strains classified as *X. c. vesicatoria.* Disparate fingerprint profiles between the four genotypes suggested that the genotypes are genetically dissimilar. Fingerprints generated from *X. c. vesicatoria* strains were unique compared to fingerprint profiles generated from more than 30 other xanthomonad pathovars or species ([24]; data not shown) and numerous strains classified as *Pseudomonas* or *Clavibacter*, as well as saprophytic bacteria associated with field tomato plants, greenhouse tomato plants, and overwintered tomato debris (data not shown).

Genotypic variation within groups A and B determined by BOX-, REP-, and ERIC-PCR. In contrast to the very different fingerprint patterns between genotypes, rep-PCR fingerprint profiles generated from DNA of strains within each genotype were highly similar (Figs. 2 and 3).

BOX-PCR differentiated 15 fingerprint types within group A. Patterns were highly similar (Fig. 2A), with differences limited to the presence or absence of one to three bands when compared to the predominant pattern highlighted by ATCC 11633 (Fig. 2A, lane 1). For example, LMG905, TS35, TS8, and Xv93-29 (Fig. 2A, lanes 2–5) each yielded a single extra prominent band of approximately 680 bp (opposing arrows in Fig. 2A, lanes 2 and 5). Likewise, strains Xcv939, Xcv931, Sp135, Sp133, Xv89, Xv334, and Xv104 (Fig. 2A, lanes 10–16) yielded a polymorphic band about 980 bp in size (opposing arrows in Fig. 2A, lanes 10 and 16). However, these seven strains were not identical. For example, most bands generated from strain Xv334 were similar to other *X. c. vesicatoria* strains, but Xv334 did not yield two bands in the 3-kb range but did yield a distinct polymorphism at 580 bp. Examples

of different polymorphisms generated from other strains are highlighted by arrowheads.

BOX-PCR delineated five distinct fingerprint patterns among strains classified as group B (Fig. 2B). The distinct BOX-PCR fingerprint patterns could not be associated with geographic region or date of isolation. For example, TS1, recovered in Ontario in 1979, and two strains recovered in Michigan (Xcv859 and Xcv736) had a BOX-PCR fingerprint indistinguishable from two Oklahoma strains (Xv10 and Xv15) (Fig. 2B, lanes 1–5). Likewise, Xcv981 and Xcv982, recovered from different Michigan fields of processing tomato in 1993, had BOX-PCR fingerprint patterns identical to strains BV6-1 and BV4-1 from Argentina and Xv56 from Brazil (Fig. 2B, lanes 15–19). Additionally, ATCC 35934, the pathovar reference strain isolated from New Zealand in 1955, could not be distinguished from five strains (DC92-13, DC92-21, DC92-23, CC164, and CC195) isolated in 1992 from independent epidemics in southwestern Ontario (Fig. 2B, lanes 6–11).

As found with BOX-PCR, ERIC-PCR patterns were highly similar, with differences limited to the presence or absence of one to three bands (Fig. 3). Using these subtle differences, ERIC-PCR differentiated 13 fingerprint types within group A. For example,

TABLE 1. Bacterial strains or DNA used in this study

Strain	Origin			Genotype/phenotype					Source/ reference[f]
	Host	Location[a]	Year	Group	Race[b]	Amylolytic[c]	Pectolytic[d]	CKTM[e]	
91W13	Pepper	ONT	1991	A	...[g]	–	–	P	R. Brammel
ATCC 11633	Pepper	NJ	1947	A	T1P2[h]	–	–	V	ATCC
LMG905	...	...	1982	A	...	...	...	...	J. Swings**
LMG910	Pepper	MOR	1976	A	...	...	...	...	J. Swings**
LMG929	Pepper	FL	1969	A	T1[h]	...	...	...	J. Swings**
P93–DIA	Pepper	GA	1993	A	...	±	–	P	G. O'Keefe
Sp124–92	Pepper	GA	1993	A	...	±	–	P	G. O'Keefe
Sp133–92	Pepper	GA	1993	A	...	–	–	P	G. O'Keefe
Sp135–92	Pepper	GA	1993	A	...	–	–	P	G. O'Keefe
Sp2–92	Pepper	GA	1993	A	...	–	–	P	G. O'Keefe
Sp66–92	Pepper	GA	1993	A	...	–	–	P	G. O'Keefe
SS–Pepper	Pepper	ONT	1992	A	...	–	–	P	B. Dhanvantari
TS8	Tomato	ONT	1990	A	T1[h]	–	±	P	B. Dhanvantari
TS16	Tomato	ONT	1990	A	...	–	±	P	B. Dhanvantari
TS26	Tomato	ONT	1990	A	...	–	±	P	B. Dhanvantari
TS31	Tomato	ONT	1990	A	T1[h]	–	–	P	B. Dhanvantari
TS35	Tomato	ONT	1990	A	T1[h]	±	–	P	B. Dhanvantari
Xv1	Pepper	FL	...	A	...	±	–	T	J. Jones
Xv18	Tomato	FL	...	A	T1P2[h]	±	–	V	J. Jones
Xv18(OH)	Pepper	OH	1992	A	P1	–	–	P	S. Miller
Xv29	Pepper	OK	1990	A	P1[h]	–	–	P	C. Bender
Xv31	Pepper	OK	1989	A	P1[h]	–	–	P	C. Bender
Xv36	...	FL	...	A	...	–	–	T	J. Jones
Xv44	Pepper	OH	1992	A	P1	–	–	P	S. Miller
Xv47	Pepper	OH	1992	A	P1	–	–	P	S. Miller
Xv63	Pepper	FL	...	A	...	±	–	P	R. Stall
Xv71	Pepper	OH	1992	A	P1	–	–	P	S. Miller
Xv75–3	...	FL	1975	A	T1	±	–	V	R. Stall*
Xv85	Tomato	FL	...	A	T1[h]	±	–	T	J. Jones
Xv88–45P	Pepper	GA	1988	A	...	–	–	P	R. Gitaitis
Xv89	Pepper	TW	...	A	P3	–	–	P	J. Jones
Xv89–52P	Pepper	GA	1989	A	...	±	–	P	R. Gitaitis
Xv89–53P	Pepper	GA	1989	A	...	–	–	P	R. Gitaitis
Xv90–1P	Pepper	GA	1990	A	...	–	–	P	R. Gitaitis
XvNC	...	GA	...	A	...	–	–	T	R. Gitaitis
Xv91	Pepper	TW	...	A	P3	–	–	V	J. Jones
Xv92–16	...	FL	1992	A	P1	–	–	P	R. Stall
Xv92–17	Pepper	FL	1992	A	P2	–	–	P	R. Stall
Xv93–1	...	FL	1993	A	P3	–	–	V	R. Stall

(*continued on next page*)

[a] Location: ONT = Ontario, NJ = New Jersey, MOR = Morocco, FL = Florida, GA = Georgia, OH = Ohio, OK = Oklahoma, TW = Taiwan, CAR = Caribbean, MX = Mexico, MI = Michigan, IN = Indiana, NZ = New Zealand, ARG = Argentina, IT = Italy, BZ = Brazil, ISR = Isreal, KS=Kansas.

[b] T1 = tomato race 1, P1 = pepper race 1, P2 = pepper race 2, and P3 = pepper race 3 (according to [28]); T2 = tomato race 2 (according to [41]).

[c] – = unable to hydrolyze starch; ± = hydrolyzed starch weakly; + = hydrolyzed starch extensively.

[d] On CVP medium: – = no activity; + = pectolytic; ± = slight activity.

[e] CKTM phenotype: P = a clear ring or "pepper type"; T = opaque white precipitate or "tomato type"; V = intermediate phenotype; – = no phenotype; ± = subtle clear ring.

[f] A. Jones, Department of Botany and Plant Pathology, Michigan State University, East Lansing; ATCC = American Type Culture Collection, Rockville, MD; B. Dhanvantari, Agriculture and Agri-Foods Canada, Harrow, Ontario; C. Bender, Department of Plant Pathology, Oklahoma State University, Stillwater; D. Cuppels, Agriculture and Agri-Foods Canada, London, Ontario; G. O'Keefe, Georgia Department of Agriculture, Tifton; J. Jones, Gulf Coast Research and Education Center, University of Florida, Bradenton; J. Swings, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; J. Tsuji, DOE–Plant Research Laboratory, Michigan State University, East Lansing; K. Dunbar, Department of Botany and Plant Pathology, Michigan State University, East Lansing; L. Afanador, Department Crop and Soil Sciences, Michigan State University, East Lansing; M. Daughtrey, Long Island Horticultural Research Laboratory, Long Island, NY; R. Brammal, Ontario Ministry of Agriculture and Food, Simcoe, Ontario, Canada; R. Stall, Department of Plant Pathology, University of Florida, Gainesville; R. Gitaitis, Department of Plant Pathology, University of Georgia, Tifton; S. Miller, Department of Plant Pathology, Ohio State University, Wooster; * = (refs. 1 and 35); ** = (refs. 37 and 38).

[g] Unknown or not determined.

[h] Race determination as reported by Bouzar et al (3).

[i] Race determination as determined in this study.

Xv18, Xv531, Xv104, and Xv334 each lacked a prominent 1.9-kb band (Fig. 3, lanes 10–13). As with BOX-PCR, Xv334 (Fig. 3, lane 13) was the least similar to other members of group A with regard to fingerprint patterns generated. ERIC-PCR fingerprinting of the group B strains showed the same five clusters showed by BOX-PCR. Figure 4A highlights a representative pattern of each cluster (lanes 1–5).

The relatively low total number of bands generated by REP-PCR rendered the REP primer set the least useful for discriminating among strains within group A. All strains within group A generated REP-PCR fingerprints identical to ATCC 11633 (Fig. 1, lane 1) with only two exceptions: Xv334 yielded two additional prominent bands, and a second collection of strains (Sp133, Sp135, Xv18-OH, Xcv931, Xcv939, and Xv89) yielded a single additional band (data not shown). REP-PCR differentiated group B strains similar to BOX- and ERIC-PCR (Fig. 4B). However, Xv56 yielded an additional band compared to BV4-1 and BV6-1 (data not shown).

Reproducibility of fingerprints. The similar rep-PCR fingerprint profiles generated from strains separated by a 40- to 50-yr period attest to the reproducibility of fingerprints generated by REP-, ERIC-, and BOX-PCR. In addition, the pathovar reference

TABLE 1. (*continued from preceding page*)

Strain	Origin			Genotype/phenotype					Source/ reference[f]
	Host	Location[a]	Year	Group	Race[b]	Amylolytic[c]	Pectolytic[d]	CKTM[e]	
Xv93–24	...	FL	1993	A	P2	–	–	–	R. Stall
Xv93–26	...	FL	1993	A	T1	–	–	T	R. Stall
Xv93–29	...	FL	1993	A	T1	–	–	T	R. Stall
Xv102	Pepper	TW	...	A	P1	–	–	P	J. Jones
Xv104	Pepper	TW	...	A	P3	±	–	P	J. Jones
Xv110	Pepper	TW	...	A	P1	–	–	P	J. Jones
Xv122	Tomato	TW	...	A	T1P2[h]	–	–	T	J. Jones
Xv300	Tomato	CAR	...	A	...	–	–	T	J. Jones
Xv334	Pepper	CAR	...	A	P1	+	–	V	J. Jones
Xv531	Tomato	CAR	...	A	P2	–	–	T	J. Jones
Xv597	Pepper	CAR	...	A	...	–	–	P	J. Jones
Xv855	Tomato	MX	...	A	P2	–	–	T	J. Jones
Xv856	Tomato	MX	...	A	P2	–	–	V	J. Jones
Xv857	Tomato	MX	...	A	P2	–	–	T	J. Jones
Xv858	Tomato	MX	...	A	P2	–	–	T	J. Jones
Xv859	Tomato	MX	...	A	P2	±	–	V	J. Jones
Xv931	Pepper	MI	1993	A	P1[1]	–	–	P	This study
Xv939	Pepper	MI	1993	A	P1[1]	–	–	P	This study
ATCC 11551	Tomato	IN	1943	B	T2P3[h]	+	+	–	ATCC
ATCC 35937	Tomato	NZ	1955	B	T2[h]	+	+	–	ATCC**
BA27–1	Pepper	ARG	...	B	T2P3	+	+	–	J. Jones*
BA29–1	Tomato	ARG	...	B	T2P3	+	+	–	J. Jones*
BV4.1	Tomato	ARG	...	B	T2	+	+	–	J. Jones
BV5–3A	Tomato	ARG	...	B	T2	+	+	–	J. Jones*
BV6.1	Tomato	ARG	...	B	T2	+	+	–	J. Jones
BV7.3A	Tomato	ARG	...	B	T2	+	+	±	J. Jones
CC164#3	Tomato	ONT	1992	B	...	+	+	–	B. Dhanvantari
CC195#1	Tomato	ONT	1992	B	...	+	+	–	B. Dhanvantari
DC92–13	Tomato	ONT	1992	B	...	+	+	–	D. Cuppels
DC92–21	Tomato	ONT	1992	B	...	+	+	–	D. Cuppels
DC92–23	Tomato	ONT	1992	B	...	+	+	–	D. Cuppels
ICBB 167	Tomato	...	...	B	T2	+	+	±	J. Jones
LMG 920	Tomato	IT	...	B	...	...	...	...	J. Swings**
TS1	Tomato	ONT	1979	B	...	+	+	–	B. Dhanvantari
Xcv736	Tomato	MI	1992	B	T2[i]	+	+	–	This study
Xcv859	Tomato	MI	1991	B	T2[i]	+	+	–	This study
Xcv981	Tomato	MI	1993	B	T2[i]	...	...	...	This study
Xcv982	Tomato	MI	1993	B	T2[i]	...	...	...	This study
Xv10	Tomato	OK	1987	B	T2[h]	+	+	±	C. Bender
Xv15	Tomato	OK	1987	B	T2[h]	+	+	–	C. Bender
Xv56	Tomato	BZ	...	B	T2	+	+	–	J. Jones*
Xv 441	Tomato	CAR	...	Outlier	P1	–	–	–	J. Jones
DC91–1	Tomato	ONT	1991	Outlier	T1[i]	+	+	T	D. Cuppels
DC92–6	Tomato	ONT	1992	Outlier	?	+	+	T	D. Cuppels
5–2–4	Geranium	ISR	1987	...	...	–	+	–	M. Daughtrey
X–1	Geranium	KS	1986	...	...	–	–	T	K. Dunbar
Xpel942	Geranium	MI	1993	...	...	–	+	–	This study
Xp805	Bean	MI	1992	...	...	+	–	V	L. Afanador
JT1	Cabbage	MI	1990	...	...	+	+	–	J. Tsuji
JT4	Arabidopsis	MI	...	...	...	+	+	T	J. Tsuji
Xcc898	Cabbage	MI	1991	...	...	+	+	–	This study
Pss11	Cherry	MI	...	...	...	–	–	–	A. Jones
Pss19	Cherry	MI	...	...	...	–	–	–	A. Jones
Pss66	Cherry	MI	...	...	...	–	–	–	A. Jones
PssI1	Cherry	MI	...	...	...	–	–	–	A. Jones
Pst915	Tomato	MI	1993	...	...	–	–	–	This study

strain was received as a culture or as DNA from three independent sources (ATCC; J. Jones, FL, and J. Swings, Belgium). Each primer set yielded identical profiles from DNA for each of the three reference strains. Likewise, duplicate samples of other strains were received over time and independently prepared and analyzed to yield identical fingerprints (data not shown). Dispensing cells directly into the PCR tubes from liquid or solid media cultures also yielded fingerprint patterns identical to patterns generated from isolated DNA (data not shown). Finally, unique bands, such as those highlighted by arrowheads in Fig. 2A, could be reproduced by independent rep-PCR experiments and the analysis of aliquots of the same PCR mixtures on agarose gels (data not shown).

Cluster analysis of *X. c. vesicatoria* strains. Because group A and B fingerprint patterns were so dissimilar, cluster analyses were performed independently on the groups. The combined data sets of BOX-, ERIC-, and REP-PCR experiments yielded 71 unique, scorable bands and were used in cluster analysis of the group A strains (data not shown). A total of 28 unique fingerprint profiles were scored. However, rep-PCR fingerprint patterns within group A were very similar, with most strains clustering at greater than 90% similarity to one another. Xv334 was an exception to this rule with a maximum of 83% similarity to any other strain. Xv334 generated unique rep-PCR fingerprint polymorphisms with each primer set compared to other strains classified within group A.

A combined data set analysis of group B yielded 64 bands, differentiating six clusters (Fig. 5). Strain-specific rep-PCR fingerprint patterns were much more distinct, with similarity values ranging from 70 to 85% compared to differences among strains in group A. Group B appears to be comprised of a more heterogeneous group of strains than group A.

Genotypic variation among nongroup A/B strains and *X. c. campestris* strains. Strains DC91-1 and DC92-6 (Fig. 1, lanes 4 and 5, 10 and 11, and 16 and 17) appeared to have several prominent bands of analogous mobility, but numerous additional bands were amplified to generate strain-specific profiles. We compared the rep-PCR fingerprint profile of these strains to those generated from other xanthomonad pathovars. We found that these two strains isolated from tomato shared several bands in common with strains of *X. c. campestris* (Fig. 6, lanes 1–4). A representative strain of *X. c. vesicatoria*, Xv29 (Fig. 6, lane 5) did not appear to share more than 1 or 2 bands with DC91-1, DC92-6 or the *X. c. campestris* strains. Several REP-PCR bands of similar mobility were also observed between DC91-1, DC92-6 and *X. c. campestris* strains (data not shown). The BOX-PCR experiments, however, provided the strongest evidence of a possible genetic relationship between DC91-1, DC92-6, and *X. c. campestris* strains.

Phenotypic characteristics of groups A and B and nongroup A/B genotypes. Because the fingerprint profiles between the dif-

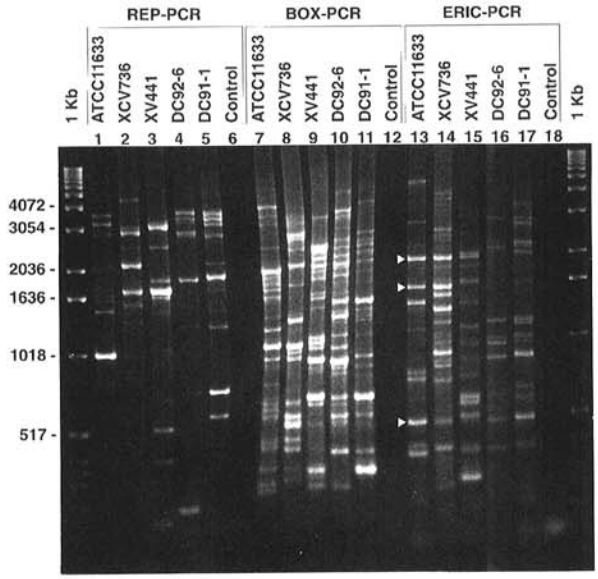

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (rep-PCR) fingerprint patterns obtained from genomic DNA from strains of *Xanthomonas campestris* pv. *vesicatoria*, using primers corresponding to repetitive extragenic palindromic sequences (REP-PCR) (lanes 1–6), BOX1A sequences (BOX-PCR) (lanes 7–12), and enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) (lanes 13–18). Six microliters of PCR products was loaded in each lane. A typical group A pattern (lanes 1, 7, and 13), group B pattern (lanes 2, 8, and 14), Xv441 pattern, (lanes 3, 9, and 15), and DC91-1 and DC92-6 patterns (lanes 4 and 5, 10 and 11, and 16 and 17) are displayed. The right and left lanes contain DNA size markers (1-kb ladder, Gibco-BRL); their sizes are indicated in base pairs. Arrowheads identify similarities or differences among selected strains. PCR bands were resolved on 1.5% agarose gels stained with ethidium bromide.

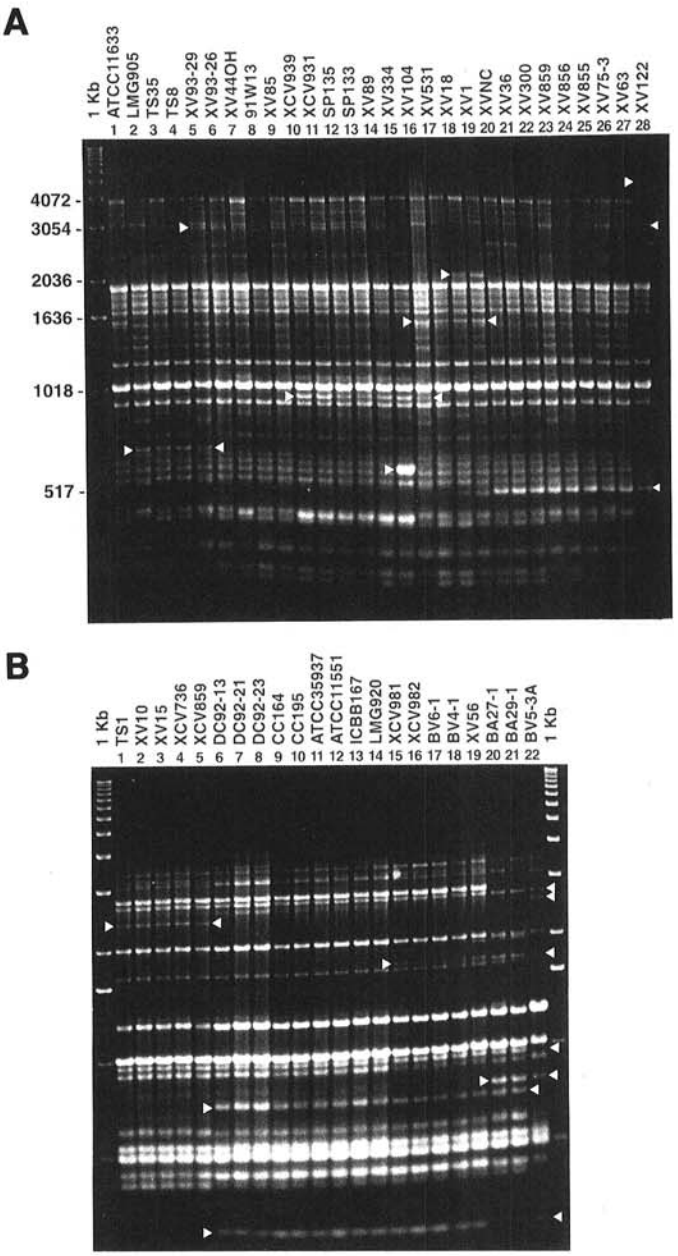

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction (rep-PCR) fingerprint patterns obtained from genomic DNA from strains of *Xanthomonas campestris* pv. *vesicatoria* **A,** group A and **B,** group B, using primers corresponding to BOX1A sequences (BOX). Arrowheads indicate polymorphisms. Six microliters of PCR products was loaded in each lane. The left lane (**A**) and right and left lanes (**B**) contain DNA size markers (1-kb ladder, Gibco-BRL); their sizes are indicated in base pairs. PCR bands were resolved on 1.5% agarose gels stained with ethidium bromide.

ferent genotypes were so distinct, we conducted several phenotypic tests to determine if specific phenotypes were associated with each genotype. Starch utilization, pectolytic activity, and cellulolytic activity are common tests to differentiate *X. c vesicatoria* from other pathogens and saprophytes in Georgia and Florida (12). CKTM is a medium selective for *X. c. vesicatoria* and is able to differentiate tomato and pepper strains (33).

Group A phenotype. Strains obtained from various parts of the world and classified within group A commonly were recovered from tomato or pepper and were nonpectolytic (Table 1). Seventy-seven percent of group A strains were starch negative, 21% hydrolyzed starch weakly, and one strain (Xv334) was starch positive (Table 1).

Within group A, 18 and 35 strains originated from tomato and pepper, respectively (Table 1). All group A strains tested, except Xv93-24, formed a precipitate on CKTM medium. Although the biochemical and genetic basis for the differential reaction on CKTM is unknown, 82% of strains isolated from pepper formed a pepper-type precipitate as described by Sijam et al (33). Nine of the strains (50%) isolated from tomato did not form a distinct tomato-type precipitate. Five of these nine strains formed a pepper precipitate on CKTM medium and came from Ontario (Ts8, Ts16, Ts26, Ts31, and Ts35). Each of these five strains was obtained from a different field in 1990, but all fields were in the same general geographic region (southwestern Ontario) (B. N. Dhanvantari, *personal communication*).

Group B phenotype. Strains classified as group B also came from various parts of the world. All strains evaluated and classified within group B hydrolyzed starch, demonstrated pectolytic activity on CVP medium, and, with one exception (BA27-1), originated from tomato (Table 1).

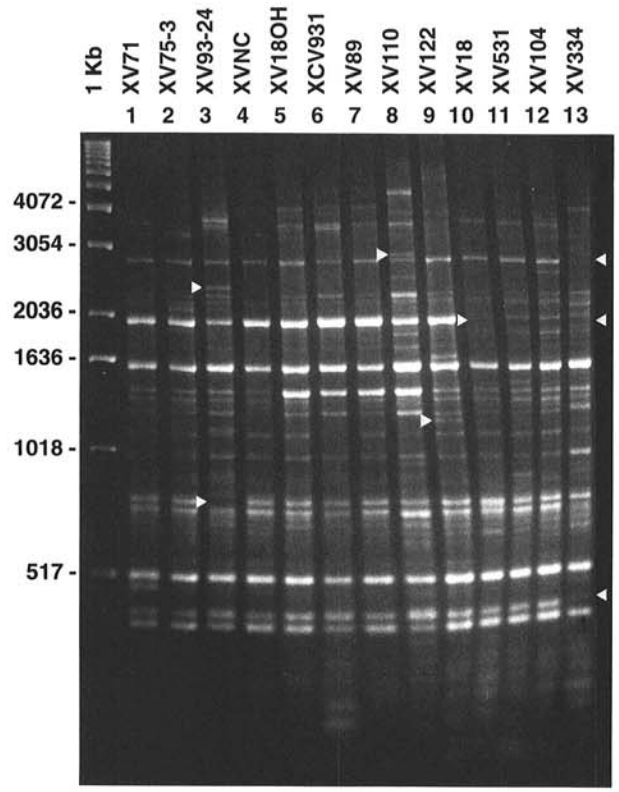

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (rep-PCR) fingerprint patterns obtained from genomic DNA from strains of *Xanthomonas campestris* pv. *vesicatoria* group A, using primers corresponding to enterobacterial repetitive intergenic consensus (ERIC) sequences. Arrowheads indicate polymorphisms. Six microliters of PCR products was loaded in each lane. The left lane contains DNA size markers (1-kb ladder, Gibco-BRL); their sizes are indicated in base pairs. PCR bands were resolved on 1.5% agarose gels stained with ethidium bromide.

Ninety-one percent of the group B strains did not form a halo on CKTM medium (Table 1). ICBB167 formed a very subtle clear ring within 3 days, and Xv10 had a light-white halo after 6 days.

Phenotype of nongroup A/B strains. Xv441 originated from tomato and was negative for both amylolytic and pectolytic activity (Table 1). Xv441 did not form a distinctive ring on CKTM (Table 1). DC91-1 and DC92-6, originated from Ontario tomato greenhouse transplants (D. Cuppels, *personal communication*), were starch and pectolytic positive, and formed a distinct tomato-type halo on CKTM media (Table 1).

All *X. c. vesicatoria* strains tested demonstrated cellulolytic activity (data not shown). Strains of *X. c. campestris* included in this study were positive for cellulolytic activity, starch hydrolysis, and pectolytic activity and formed a tomato-type precipitate or no precipitate on CKTM media (Table 1). Other pathovars of *X. c. campestris* were cellulolytic positive and were negative or positive for starch hydrolysis and pectolytic activity (Table 1). *Pseudomonas* strains effectively functioned as controls and were negative for cellulolytic activity, starch hydrolysis, and pectolytic activity (Table 1).

Pathogenicity and race determination. Each described known race, *sensu* Minsavage et al (27), including tomato race 1 (T1 of

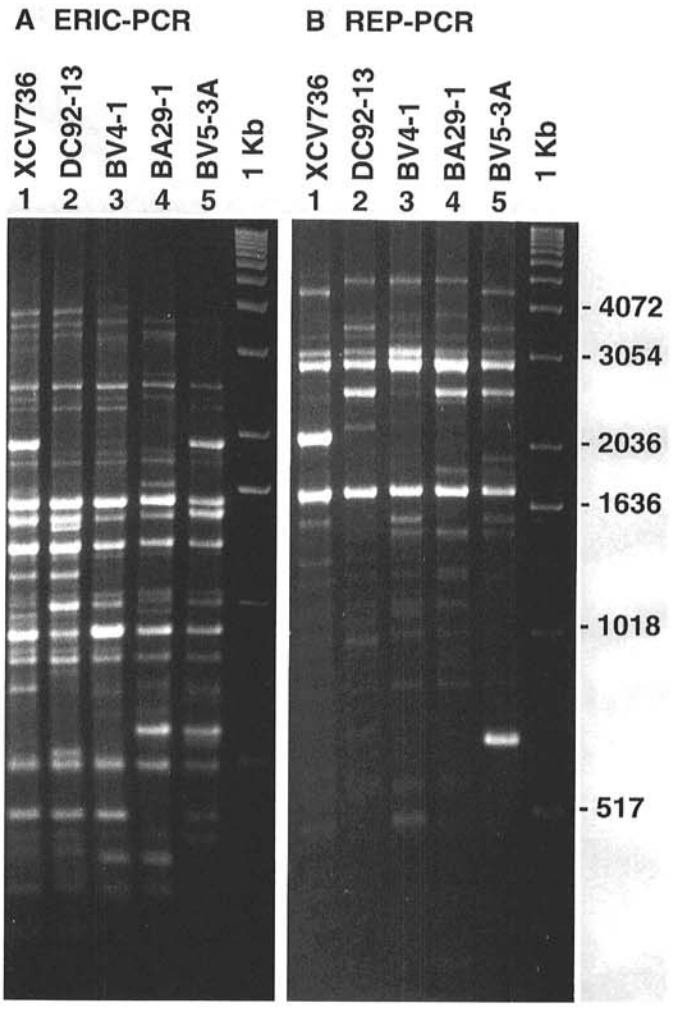

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction (rep-PCR) fingerprint patterns obtained from genomic DNA from strains of *Xanthomonas campestris* pv. *vesicatoria* group B, using primers corresponding to **A,** enterobacterial repetitive intergenic consensus (ERIC-PCR) sequence and **B,** repetitive extragenic palindromic sequence (REP-PCR) primers. Six microliters of PCR products was loaded in each lane. The right lanes contain DNA size markers (1-kb ladder, Gibco-BRL); their sizes are indicated in base pairs. PCR bands were resolved on 1.5% agarose gels stained with ethidium bromide.

the *X. c. vesicatoria* T group), pepper race 1 (of the *X. c. vesicatoria* P group), pepper race 2 (of the *X. c. vesicatoria* PT group), and pepper race 3 (of the *X. c. vesicatoria* PT group) had genomic rep-PCR fingerprints characteristic of group A (Figs. 2A and 3A). Race designation of strains within group A could not be correlated to total chromosomal fingerprint patterns as determined by rep-PCR. Strain Xv441 from the Caribbean Islands has been classified as pepper race 1 (H. Bouzar, J. Jones, and R. E. Stall, University of Florida, *personal communication*). We confirmed its race designation and noted extended water-soaking (i.e., pathogenicity) after infiltration of colonies into the leaf intercellular space of the pepper line Early Cal-Wonder.

One to four infiltration and spray-inoculation tests with tomato were conducted with DC91-1, DC92-6, and selected strains of groups A and B. Group A strains, including Xv93-26, Xv75-3, Xcv931, and Xcv939, were virulent for Bonny Best but not Hawaii 7998. Strains classified as tomato race 2 (*sensu* Wang et al [40]) belonged to group B, which is consistent with the results obtained by Bouzar et al (3). Representative strains classified as group B based on rep-PCR, including ATCC 35937, Xv56, and the Michigan strains Xcv736, Xcv981, and Xcv982, were virulent for both Bonny Best and Hawaii 7998. In spray-inoculation tests, symptoms incited by group B strains became apparent within 7 days and consisted of numerous leaf spots. Spots were necrotic, irregular, often coalescent, and generally restricted to the foliage. In contrast, DC 91-1 also was virulent on Bonny Best and incited symptoms atypical for other *X. c. vesicatoria* group B strains. Distinct circular necrotic lesions formed on foliage within 4–5 days postinoculation. After two to three additional days, entire leaflets began to wilt. DC 91-1 also incited large elliptical lesions on petiole and stem tissue. Lesions enlarged and on occasion girdled the entire stem, resulting in complete wilt of apical tissues. DC 91-1 was not virulent on Hawaii 7998. DC 92-6 simply incited chlorotic flecking on Bonny Best and was not virulent on Hawaii 7998 after spray-inoculation. Strains classified as T1, e.g., Xv93-26, incited lesions on tomato similar to DC91-1.

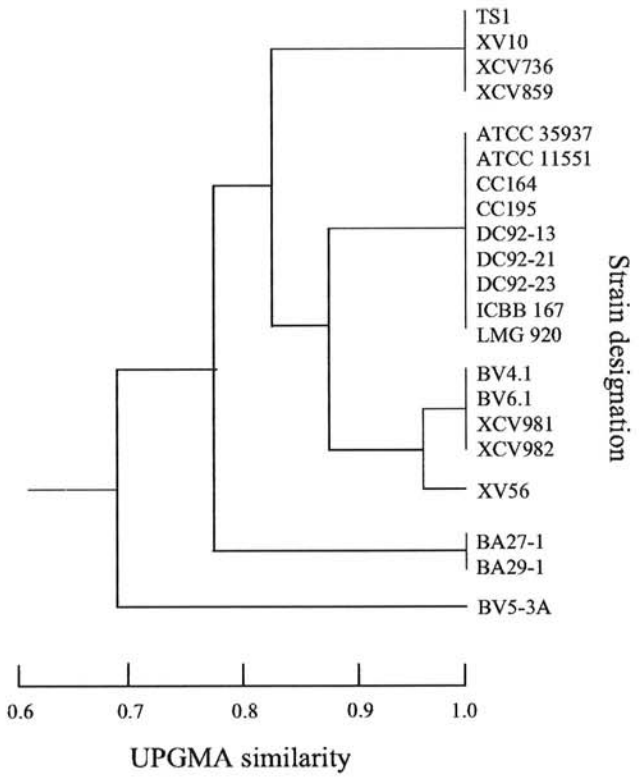

Fig. 5. Cluster analysis of strains of *Xanthomonas campestris* pv. *vesicatoria* classified as group B based on the presence and absence of bands generated using primers corresponding to repetitive extragenic palindromic sequences (REP), BOX1A sequences (BOX), and enterobacterial repetitive intergenic consensus sequences (ERIC) in polymerase chain reaction.

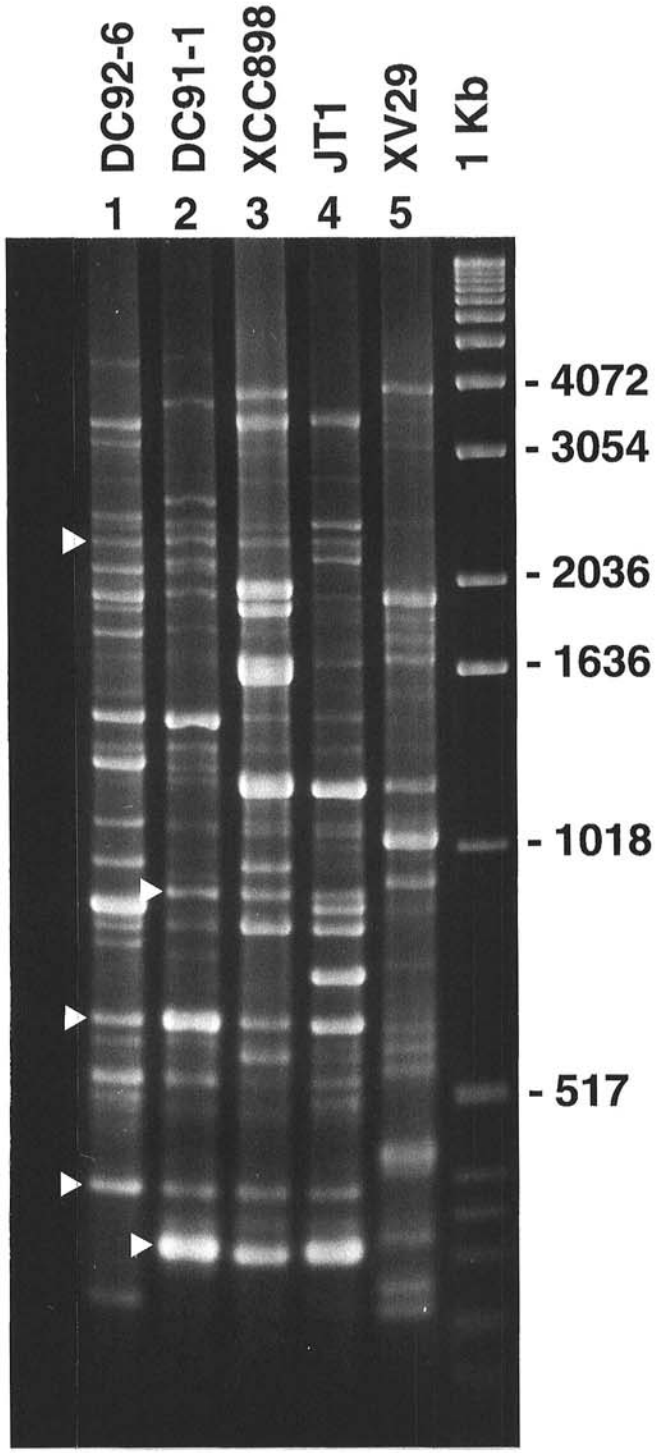

Fig. 6. Agarose gel electrophoresis of polymerase chain reaction (rep-PCR) fingerprint patterns obtained from genomic DNA from nongroup A or B strains of *Xanthomonas campestris* pv. *vesicatoria* (lanes 1 and 2) compared to patterns generated from representative strains of *X. c. campestris* (lanes 3 and 4) and group A strains of *X. c. vesicatoria* (lane 5), using BOX1A sequence (BOX) primers. Arrowheads indicate bands of similar size. Six microliters of PCR products was loaded in each lane. The right lane contains DNA size markers (1-kb ladder, Gibco-BRL); their sizes are indicated in base pairs. PCR bands were resolved on 1.5% agarose gels stained with ethidium bromide.

DISCUSSION

In this paper, we confirmed and extended our earlier observations with regard to the utility of rep-PCR–mediated genomic fingerprinting of bacteria (Versalovic et al [39]). Of particular importance with regard to bacteria associated with plants is that rep-PCR is able to differentiate symbiotic or pathogenic bacteria at the species, pathovar/biovar, or strain level (6,21,23,24,39; F. J. Louws, M. Schneider, D. W. Fulbright, and F. J. de Bruijn, *unpublished data*). Based on this study, strains classified as *X. c. vesicatoria* clearly fell into four genotypes. With the exception of three strains, the majority of strains belonged to group A or B. Strains within group A were highly homogenous to one another, and strains within group B were more diverse genetically. Each primer set successfully differentiated the four genotypes, but the combined data provided a more detailed assessment of the chromosomal structure and strain diversity compared to data generated by one primer set alone.

X. c. vesicatoria has been described as a pathovar comprised of diverse strains (2–4,8,20,22,27,34,36,37,40). This report highlights the complexity of the observed diversity based on various phenotypic features. However, in this study we have been able to categorize such diversity within a useful genotypic framework as determined by rep-PCR. For example, *X. c. vesicatoria* has been described as pectolytic or nonpectolytic (1) with the ability (9), inability (12), or variable ability (3,8) to hydrolyze starch. Our work confirms other reports (3,34) that amylolytic and pectolytic activities are highly associated with specific genotypes. More than 96% of the strains evaluated in this study were classified as group A or B, and the strains in each group were predominantly amylolytic/pectolytic minus or weak, and amylolytic/pectolytic plus, respectively.

The importance of discerning genetic diversity is highlighted by our findings that group B strains comprise an important component of the tomato bacterial spot complex in the Northcentral tomato production region of North America. Our results indicate that *X. c. vesicatoria* strains with amylolytic and pectolytic activity are more numerous and widely distributed than previously thought. Most work with *X. c. vesicatoria* has been conducted in Florida and Georgia where the majority of strains are diagnostically unable to hydrolyze starch and are nonpectolytic (1,12,34). Beaulieu et al (1) concluded pectolytic activity was correlated with the geographic origin of isolation, since 90% of strains from Argentina had pectolytic activity (including strains used in our study, such as Xv 56, BV5-3a, and BA27-1) compared to 0.003% from the United States. Bouzar et al (3) recently documented the worldwide distribution of both group A and B strains, and our study extends their analysis. In addition to the strains from Indiana (ATCC 11551) and Oklahoma (Xv10 and Xv15), we have analyzed strains from field tomatoes or greenhouse transplants obtained from more than 14 sites in Ontario and Michigan (including DC92-13, DC92-21, DC92-23, TS1, CC164#3, and CC195#1 and Xcv859, Xcv736, Xcv981, and Xcv982); all the strains belonged to group B.

We noted polymorphisms among group A and B strains using REP-, BOX-, and ERIC-PCR. Within group A, polymorphisms were simple (with differences limited to one to three DNA bands with any given primer set), with the exception of Xv334. Xv334 was polymorphic with all three primer sets. Xv334 also was physiologically atypical because it had starch hydrolytic activity (H. Bouzar, J. B. Jones, and R. E. Stall, University of Florida, *personal communication*; this study). In contrast, six distinct patterns or lineages could be elucidated by each primer set within group B. Stall et al (34) also concluded that group B appears to comprise a more heterogeneous collection of strains compared to group A.

The group A and B subpopulations of *X. c. vesicatoria* have recently been independently described by others (2,3,34,37), providing a large database of the characteristics of each group. We performed rep-PCR on numerous strains that have been included in each of the four studies, and our observations using the simple, rapid procedure of rep-PCR are consistent with the polyphasic approaches published by others.

Numerous correlations of rep-PCR with other taxonomic approaches is of interest, and we highlight a few here. Bouzar et al (3) noted that the carbon utilization ability of different strains classified as *X. c. vesicatoria* is as diverse as various pathovars of *X. campestris.* Carbon utilization patterns did not effectively group all A or B strains together, compared to other xanthomonad pathovars (3). Our study included eight of 11 phena determined by Bouzar et al (3), and rep-PCR effectively grouped all A and B strains into distinct groups (this study) compared to other pathovars (24). Likewise, cellular composition of fatty acids did not group A and B strains into two distinct phena after computation of Euclidean distance to each strain (3), in contrast to the data generated with rep-PCR (this study).

Bouzar et al (2) also demonstrated that groups A and B each have a diagnostic protein, designated as alpha and beta, respectively. They were able to group 252 *X. c. vesicatoria* strains as group A, group B, or "unusual phenotype" based on the alpha and beta bands, amylolytic activity, and pectolytic activity. They noted that strains 8, 31, and 35 from Canada (also TS8, TS31, and TS35) displayed an unusual phenotype with an alpha band but expressing pectolytic activity. In our study, these three strains clearly had a group A genotype. However, we also observed pectolytic activity, and these strains, although isolated from tomato, expressed a pepper-strain phenotype on CKTM medium. Thus, biochemical phenotypes may be inconsistent within groups, but the alpha band (2) and rep-PCR effectively classified these strains as group A.

Also in this study, within group B identical rep-PCR fingerprint profiles from genomic DNA of BA27-1 and BA29-1 were generated, and this profile differed from BV5-3A and Xv56. Stall et al (34) also used these strains in their study and showed that monoclonal antibodies distinguished BA27-1 and BA29-1 compared to BV5-3A and Xv56. Based on restriction digest analysis, Stall et al (34) also noted that BV27-1 and BV29-1 have a very small genetic distance, whereas Xv56 and BV5-3A have a greater genetic distance, further corroborating the results of this study.

The fact that strains classified as groups A and B are so dissimilar (34, 37, this study) suggests that pathogenicity for tomatoes occurred through convergent evolution and that the population structure of *X. c. vesicatoria* is polyphyletic. The importance of the nongroup A/B genotypes, Xv441, DC91-1 and DC92-6, and the presence of other genotypes pathogenic to tomato and/or pepper is unknown. Xv441 was discovered in the Caribbean region and has been noted as unique based on polyphasic phenotypic experiments (H. Bouzar, J. B. Jones, and R. E. Stall, University of Florida, *personal communication*). DC91-1 is a highly virulent and destructive pathogen based on our pathogenicity tests and economic damage observed in a commercial greenhouse (D. Cuppels, Agriculture Canada, London, Ontario, *personal communication*). DC92-6 also was recovered from tomato seedlings in the greenhouse; it shared numerous comigrating bands similar to DC91-1, but did not appear to be highly virulent to tomato. The detection of these genotypically distinct strains (DC91-1 and DC92-6) invites numerous questions on the origin of these strains, the genetic differences between the highly virulent strain (DC91-1) and the less virulent strain (DC92-6), and the potential of the highly virulent strain to become a predominant clone. Initial work demonstrating common bands between DC91-1, DC92-6, and strains representative of *X. c. campestris* suggests these *X. c. vesicatoria* strains from Ontario may have originated from or have a common ancestry with *X. c. campestris.* Additional sampling and evaluation may in fact reveal other genotypes able to incite bacterial spot of tomato.

Knowledge of the population structure of *X. c. vesicatoria* will aid in the selection of representative strains for taxonomic analy-

ses, for evolutionary, ecological, and epidemiological studies, and for devising integrated disease management strategies of bacterial spot of tomato, such as diagnostic, detection, and plant-breeding programs.

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