Genetic Diversity of *Xanthomonas campestris* pv. *vitians*, the Causal Agent of Bacterial Leafspot of Lettuce

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ABSTRACT

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Bacterial leafspot of lettuce (BLS), caused by *Xanthomonas campestris* pv. *vitians*, has become more prevalent in many lettuce-growing areas of the world over the past decade. To gain insight into the nature of these outbreaks, the genetic variation in *X. campestris* pv. *vitians* strains from different geographical locations was examined. All strains were first tested for pathogenicity on lettuce plants, and then genetic diversity was assessed using (i) gas-chromatographic analysis of bacterial fatty acids, (ii) polymerase chain reaction analysis of repetitive DNA sequences (rep-PCR), (iii) DNA sequence analysis of the internal transcribed spacer region 1 (ITS1) of the ribosomal RNA, (iv) restriction fragment length polymorphism (RFLP) analysis of total genomic DNA

with a repetitive DNA probe, and (v) detection and partial characterization of plasmid DNA. Fatty acid analysis identified all pathogenic strains as *X. campestris*, but did not consistently identify all the strains as *X. campestris* pv. *vitians*. The rep-PCR fingerprints and ITS1 sequences of all pathogenic *X. campestris* pv. *vitians* strains examined were identical, and distinct from those of the other *X. campestris* pathovars. Thus, these characteristics did not reveal genetic diversity among *X. campestris* pv. *vitians* strains, but did allow for differentiation of *X. campestris* pathovars. Genetic diversity among *X. campestris* pv. *vitians* strains was revealed by RFLP analysis with a repetitive DNA probe and by characterization of plasmid DNA. This diversity was greatest among strains from different geographical regions, although diversity among strains from the same location also was detected. The results of this study suggest that these *X. campestris* pv. *vitians* strains are not clonal, but comprise a relatively homogeneous group.

Over the past decade, outbreaks of bacterial leafspot (BLS) of lettuce, caused by *Xanthomonas campestris* pv. *vitians*, have occurred with increasing frequency throughout the world. Recent outbreaks have been reported from the United States (e.g., California [1], Florida [27], Hawaii [19], and Ohio [30]), Italy (43), Venezuela (9), Canada (6), and Turkey (29). Although *X. campestris* pv. *vitians* is widely distributed, little is known of the genetic diversity of this pathogen.

A number of approaches can be used to examine the genetic diversity of members of the genus *Xanthomonas* (5,11,13,20,21,24,34,35,37-41). Vauterin et al. (39) analyzed protein electrophoresis patterns of 900 strains of *X. campestris* (Dye) and found that most, but not all, pathovars could be differentiated. Whereas most pathovars were comprised of a relatively homogeneous group of strains, some pathovars, including *X. campestris* pv. *vitians*, contained aberrant strains (39). Of the 10 strains of *X. campestris* pv. *vitians* from six countries (Colombia, Zimbabwe, United States, New Zealand, Brazil, and Australia) that were examined in this study, 8 had similar protein patterns, but the pathovar reference strain from Colombia (LMG 937) and a strain from Australia (LMG 8689) had patterns that clearly were distinct from each other and from those of the other *X. campestris* pv. *vitians* strains. Stefani et al. (35) also used protein electrophoresis patterns to examine genetic diversity among strains of *X. campestris* pv. *vitians* and other *X. campestris* pathovars. In this study, the 21 strains of *X. campestris* pv. *vitians* examined (from New Zealand, United States, Brazil, Zimbabwe, and Italy) composed a fairly homogeneous group, despite their wide geographical distribution. The *X. campestris* pv. *vitians* strains also were differentiated from other pathovars including *campestris*, *vesicatoria*, *pelargonii*,

phaseoli, and *juglandis*, as well as *Xanthomonas*-like saprophytes recovered from lettuce. However, the pathovar reference strain (NCPPB 976=LMG 937) was considered to be aberrant based on having distinct colony morphology and protein pattern and, significantly, it was not pathogenic to lettuce (35).

Vauterin et al. (37) performed a comprehensive analysis of 183 strains of *X. campestris* using DNA hybridization data as well as other properties (phenotypic data from previous studies and metabolic activity on various carbon substrates), and proposed a new classification system for the genus *Xanthomonas*. On the basis of these analyses, it was concluded that *X. campestris* pv. *vitians* was a heterogeneous pathovar composed of two groups, type A and B. Type A included only the pathovar reference strain (LMG 937) and was named *X. axonopodis* pv. *vitians*. Type B contained all other strains, including LMG 8689 (previously reported to be an aberrant strain based on protein electrophoresis patterns) (39), and was named *X. hortorum* pv. *vitians*.

In the present study, the genetic diversity of *X. campestris* pv. *vitians* strains associated with recent BLS outbreaks of lettuce in California as well as those from a number of other geographical locations were examined. Five methods were used: (i) gas chromatographic (GLC) analysis of bacterial fatty acids, (ii) sequence analysis of the internal transcribed spacer region 1 (ITS1) of the ribosomal RNA, (iii) polymerase chain reaction analysis of repetitive DNA sequences (rep-PCR), (iv) restriction fragment length polymorphism (RFLP) analysis of total genomic bacterial DNA with a repetitive DNA probe, and (v) detection and partial characterization of plasmid DNA. The results of these analyses suggested that these strains compose a relatively homogeneous group, and did not reveal distinct groups or aberrant strains.

MATERIALS AND METHODS

Bacterial strains. The sources, year of isolation, and geographical origin of the strains *X. campestris* pv. *vitians* strains

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examined in this study are shown in Table 1. Strains from California were isolated from lettuce plants with BLS symptoms or symptomless weeds growing near lettuce fields (1). One strain was recovered from a contaminated seed lot from an unknown location. Strains from other geographical locations were provided by other investigators or were obtained from the Laboratorium voor Microbiologie Gent Culture Collection (Gent, Belgium). All strains were subcultured by streaking on 523 medium (16), and single colonies were isolated. A single representative colony was selected, grown in yeast tryptone (YT) broth, and stored in 80% glycerol at -80°C . These frozen stock cultures served as sources of bacteria for subsequent experiments.

Pathogenicity tests. All putative strains of *X. campestris* pv. *vitians* were tested for pathogenicity on leaves of lettuce plants. Bacterial cells from a single colony of the strain to be tested were added to 2 ml of YT broth in a 5-ml polypropylene tube (Fisher Scientific, Pittsburgh). Tubes were placed on a rotary shaker and cultures were grown for 30 h at 28°C . Cultures were adjusted to an optical density of 0.4 at 600 nm (approximately 10^8 CFU/ml) and carborundum was added. The bacterial cell suspensions were rub inoculated with cotton swabs onto leaves of 3-week-old lettuce plants (cv. Salinas MD). The positive control was leaves inoculated with a California strain of *X. campestris* pv. *vitians* (strain Sal) (Table 1), whereas the negative control was leaves inoculated with sterile YT and carborundum. Plants were kept in a greenhouse maintained at approximately 28 to 30°C , and examined 7 and 10 days postinoculation (dpi) for development of disease symptoms.

Fatty acid analysis. Strains of *X. campestris* pv. *vitians* used for fatty acid analyses (Table 1) were recovered from stock cultures by streaking onto 523 medium. Strains then were grown on trypticase soy broth agar (Fisher Scientific) for 24 h at 28°C . Fatty acids were prepared according to the manufacturer's instructions, and whole cell fatty acid analysis was carried out by gas chromatography (26). *Stenotrophomonas maltophilia* was included as a positive control. Fatty acid profiles were determined for each strain and compared with available reference strains in the aerobe database of the Microbial Identification System (MIS) software package (version 3.8, September 1994 update; MIDI, Inc., Newark, DE).

Extraction of DNA. The strains used for rep-PCR, ITS1 sequence, and RFLP analyses are indicated in Table 1. Bacterial cells from a single colony were taken from a 24-h-old bacterial streak culture on 523 medium and added to 2 ml of YT broth in a 5-ml polypropylene tube. The tubes were placed on a rotary shaker and cultures were grown for 24 h at 28°C . Nucleic acids were extracted from bacterial cells by the cetyltrimethylammonium bromide method (17,31) and suspended in 100 μl of sterile water. DNA concentrations were determined with a fluorometer (Model TKO 100; Hoefer Scientific Instruments, San Francisco). For PCR amplification of the ITS1 region and rep-PCR analyses, DNA was adjusted to a final concentration of 10 ng/ μl with sterile water.

Rep-PCR. Rep-PCR was performed with the following primers: repetitive extragenic palindromic (REP)-PCR, 1R and 2I; enterobacterial repetitive intergenic consensus (ERIC)-PCR, 1R and 2; and BOX element 1A (BOX)-PCR, 1R (28). The PCR was carried out as described by Rademaker and de Bruijn (28) with 20 ng of total genomic DNA. PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA), and PCR products were examined by electrophoresis in 0.7% agarose gels in 0.5 $\times$ Tris-borate EDTA (TBE) buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel imaging system.

PCR amplification and sequence analysis of the ITS1 region. The ITS1 rRNA spacer region, which lies between the 16S and 23S rRNA genes, was amplified from 20 ng of total genomic DNA by PCR with the G1/L1 primer pair (15). The PCR was per-

formed in a GeneAmp 2400 (Perkin-Elmer Cetus), and PCR products were examined by electrophoresis in 1% agarose gels in Tris-acetate EDTA (TAE) buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel imaging system (Alpha Innotech Corp., San Leandro, CA). PCR-amplified DNA fragments were recovered using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations.

PCR-amplified DNA fragments were sequenced directly using ABI PRISM Dye Terminator Cycle Sequencing with dRhodamine terminator chemistry. Sequencing reactions were fractionated in 5% Long Ranger gels on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA). Sequence data were analyzed using ABI Prism $\text{\AA}$ Sequencing 2.1.1 software (Perkin-Elmer Cetus) and DNA sequence comparisons were done with the software of the Genetics Computer Group, version 10, University of Wisconsin-Madison (10). The ITS1 sequences of strains of *X. campestris* were aligned using the Clustal W subprogram available in MacVector (version 7.1, 2001; Accelrys, Inc., Burlington, MA). Multiple alignment parameters were set to open, extended gap penalties were 2 and 1, respectively, and transitions were unweighted. No ambiguities were found in this alignment. The outgroup taxon, *Xylella fastidiosa*, was selected based on a BLAST search of the NCBI database using *Xanthomonas campestris* pv. *vitians* strain Sal as the template sequence. This search served to provide a taxon having the highest degree of sequence similarity outside the genus *Xanthomonas*. A non-*Xanthomonas* outgroup taxon was used to obtain a valid polarization of character traits in the phylogenetic analysis. The *Xylella fastidiosa* sequence was aligned to the *Xanthomonas campestris* alignment by hand based on secondary structure of rRNA (25).

Phylogenetic affiliations were inferred based on maximum parsimony using the computer program PAUP (version 4.0b10; Altivec) for the Macintosh (36). Parsimony analysis was conducted on informative characters using only a heuristic search having the random stepwise addition option. Gaps were set as missing data with transitions and transversion unweighted. Branch support was estimated by bootstrap analysis of 1,000 replications. Clade support was characterized using the computer program MacClade (version 3.05) to trace unambiguous synapomorphies (22).

RFLP analysis. Approximately 2 μg of total genomic DNA was digested with *EcoRI*, *BamHI*, or *HincII* in a 50- μl reaction (New England BioLabs, Beverly, MA). Digested DNA (approximately 1 μg) was fractionated in 0.7% agarose gels in 0.5 $\times$ TBE buffer, gels were stained with ethidium bromide, and DNA was visualized with a gel imaging system. DNA was transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England) and hybridized with a repetitive probe as previously described (12,31). The probe was the recombinant plasmid P2, which contains a 3.4-kb genomic DNA fragment from *X. campestris* pv. *phaseoli* (13), labeled with [α - ^{32}P] dCTP by nick translation (Life Technologies, Grand Island, NY). Membranes were hybridized with a probe activity of approximately 1.2 decays per minute/ml of hybridization buffer. Hybridization was conducted in 30% formamide, 0.125 M Na_2HPO_4 (pH 7.2), 2% sodium dodecyl sulfate (SDS), and 1 mM EDTA (pH 8.0) overnight at 37°C in a hybridization oven (model HB-1D; Techne, Princeton, NJ). Membranes were washed twice in 0.125 M Na_2HPO_4 (pH 7.2), 1 mM EDTA (pH 8.0), and 2% SDS at 42°C for 15 min; and once in 0.0075 M Na_2HPO_4 (pH 7.2), 1 mM EDTA (pH 8.0), and 2% SDS at 42°C for 15 min. Membranes then were exposed to X-ray film (Fuji, Stamford, CT) for 12 and 24 h.

Detection and analysis of plasmid DNA. Bacterial cells from a single colony were taken from a 24-h-old streak culture on 523 medium and added to 50 ml of Luria broth in a 150-ml Erlenmeyer flask. Flasks were placed on a rotary shaker and cultures were grown for 24 h at 28°C . Plasmid DNA was extracted from

TABLE 1. *Xanthomonas campestris* strains used for fatty acid analysis, rDNA sequencing, polymerase chain reaction analysis of repetitive DNA sequences (rep-PCR), restriction fragment length polymorphism (RFLP) analysis, and plasmid profiling

<i>X. campestris</i> strain	Year	Host	Geographical origin	Fatty acid	ITS1 ^a	rep-PCR	RFLP	Plasmid
<i>pv. vitians</i>								
Fl-1	1995	<i>Lactuca sativa</i>	Florida	+	...	...	+	+
9301	1993	<i>Lactuca sativa</i>	Gonzales, CA	+	...	...	...	...
9302	1993	<i>Lactuca sativa</i>	Salinas, CA	+	...	+	...	...
9303	1993	<i>Lactuca sativa</i>	Gonzales, CA	+	AF279425	...	...	...
9304	1993	<i>Lactuca sativa</i>	Chualar, CA	+	...	...	...	...
9305	1993	<i>Lactuca sativa</i>	Guadalupe, CA	+	...	...	...	...
9306	1993	<i>Lactuca sativa</i>	Salinas, CA	+	...	...	...	...
9307	1993	<i>Lactuca sativa</i>	Salinas, CA	+	...	...	...	...
9308	1993	<i>Lactuca sativa</i>	Gonzales, CA	+	...	+	...	...
9309	1993	<i>Lactuca sativa</i>	Salinas, CA	+	...	...	...	...
9310	1993	<i>Lactuca sativa</i>	Salinas, CA	...	...	+	...	...
H+	1995	Seed	Unknown location	+	...	...	...	...
41335	1983	<i>Lactuca sativa</i>	Australia	+	AF279426	+	+	+
30547	1978	<i>Lactuca sativa</i>	Pimlico, NSW, Australia	+	...	+	+	...
31975	1978	<i>Lactuca sativa</i>	Dareton, NSW, Australia	+	...	+	+	+
30526	1978	<i>Lactuca sativa</i>	Rydalmere, NSW, Australia	+	AF279427	+	+	...
95-02	1995	<i>Lactuca sativa</i>	Watsonville, Ca	+	...	...	...	...
Pyb251	1995	<i>Lactuca sativa</i>	Monterey, CA	+	...	+	...	...
Sal	1995	<i>Lactuca sativa</i>	Salinas, CA	+	AF279422	+	+	+
KC	1995	<i>Lactuca sativa</i>	King City, CA	+	...	...	...	...
GON	1995	<i>Lactuca sativa</i>	Gonzales, CA	+	...	...	...	...
9401	1994	<i>Lactuca sativa</i>	Salinas, CA	+	...	...	...	...
B162	1995	<i>Lactuca sativa</i>	Guadalupe, CA	+	AF279424	...	+	+
LL2	1995	<i>Lactuca sativa</i>	Bakersfield, CA	+	AF279423	...	+	+
9812	1998	<i>Lactuca sativa</i>	Salinas, CA	...	...	+	...	...
9862	1998	<i>Senecio vulgaris</i>	Salinas, CA	...	+	+	...	...
9868	1998	<i>Lactuca sativa</i>	Salinas, CA	...	...	+	+	...
9873	1998	<i>Lactuca sativa</i>	Salinas, CA	...	...	+	...	...
9879	1998	<i>Lactuca sativa</i>	Salinas, CA	...	...	+	+	...
9813	1998	<i>Senecio vulgaris</i>	Santa Maria, CA	...	...	+	...	...
9815	1998	<i>Malva parviflora</i>	Santa Maria, CA	...	+	+	...	...
9824	1998	<i>Sonchus oleraceus</i>	Santa Maria, CA	...	+	+	+	+
9839	1998	<i>Lactuca sativa</i>	Santa Maria, CA	...	...	+	...	...
9844	1998	<i>Lactuca sativa</i>	Santa Maria, CA	...	...	+	+	...
9832	1998	<i>Brassica rapa</i>	Lompoc, CA	...	...	+	...	...
9828	1998	<i>Lupinus formosus</i>	Lompoc, CA	...	...	+	...	...
9834	1998	<i>Malva parviflora</i>	Lompoc, CA	...	...	+	...	...
9851	1998	<i>Polygonum arenastrum</i>	Lompoc, CA	...	+	+	+	...
9856	1998	<i>Lactuca sativa</i>	Lompoc, CA	...	...	+	+	...
9906	1979	<i>Lactuca sativa</i>	Kula, HI	...	...	+	...	...
9909	1979	<i>Lactuca sativa</i>	Kula, HI	...	...	+	...	...
9910	1979	<i>Lactuca sativa</i>	Kula, HI	...	...	+	...	...
9912	1979	<i>Lactuca sativa</i>	Kula, HI	...	+	+	+	+
9915	1979	<i>Lactuca sativa</i>	Kula, HI	...	+	...	...	...
9916	1979	<i>Lactuca sativa</i>	Kula, HI	...	...	+	+	+
9918	1979	<i>Lactuca sativa</i>	Kula, HI	...	...	+	...	...
LMG ^b 937 ^c	1961	<i>Lactuca sativa</i>	Columbia	...	+	+	...	...
LMG 8689	1988	<i>Lactuca sativa</i>	Australia	...	+	+	...	...
<i>pv. alfalfae</i>								
101	1995	<i>Medicago sativa</i>	California	...	...	+	...	...
102	1995	<i>Medicago sativa</i>	California	...	...	+	...	...
<i>pv. armoraciae</i>								
275	1992	<i>Brassica oleracea</i>	California	...	...	+	...	...
756	1992	<i>Brassica oleracea</i>	California	...	...	+	...	...
<i>pv. campestris</i>								
295	1993	<i>Brassica oleracea</i>	California	...	...	+	...	...
404	1994	<i>Brassica oleracea</i>	California	...	...	+	...	...
<i>pv. carotae</i>								
4	1994	<i>Daucus carota</i>	Davis, CA	...	AF279430	...	...	...
16	1995	<i>Daucus carota</i>	Cuyama, CA	...	AF279428	...	...	...
21	1995	<i>Daucus carota</i>	Owens Valley, CA	...	AF279429	...	...	...
9936	1999	<i>Daucus carota</i>	Lancaster, CA	...	...	+	...	...
9940	1999	<i>Daucus carota</i>	Lancaster, CA	...	...	+	...	...
<i>pv. phaseoli</i>								
17	1997	<i>Phaseolus vulgaris</i>	Malawi	...	AF279431	...	...	...
20	1998	<i>Phaseolus vulgaris</i>	Tanzania	...	AF279430	...	...	...
<i>var. fuscans</i>								
12	1997	<i>Phaseolus vulgaris</i>	Malawi	...	AF279433	...	...	...
13	1997	<i>Phaseolus vulgaris</i>	Malawi	...	...	+	...	...
16	1997	<i>Phaseolus vulgaris</i>	Malawi	...	AF279434	...	...	...
54	1987	<i>Phaseolus vulgaris</i>	Brazil	...	...	+	...	...

^a ITS = internal transcribed spacer region 1 sequence; accession numbers for sequences deposited in the GeneBank.

^b LMG = Culture Collection, Laboratorium voor Microbiologie, Gent, Belgium; + indicates that a strain was used for this analysis.

^c Pathovar reference strain.

bacterial cells with the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA). Plasmid DNA was analyzed by agarose gel (0.5%) electrophoresis in 0.5% TAE, and the concentration of each plasmid DNA preparation was determined with a fluorometer. Approximately 100 ng of plasmid DNA was digested with *Eco*RI, *Bam*HI, or *Sma*I in a 25- μ l reaction (New England BioLabs, Beverly, MA). Digested plasmid DNA was analyzed by agarose gel electrophoresis in 0.5% gels in 0.5 $\times$ TBE buffer. Gels were stained with ethidium bromide and DNA was visualized with a gel imaging system.

RESULTS

Pathogenicity tests. Strains of *X. campestris* pv. *vitians* induced dark, brown-black watersoaked lesions with yellow margins on inoculated lettuce leaves approximately 7 to 10 dpi. Pathogenic strains of *X. campestris* pv. *vitians* were recovered from lettuce leaves with BLS symptoms, from leaves of symptomless weeds from various locations in California, and from a contaminated seed lot. Additional pathogenic strains were obtained from Florida, Hawaii, and Australia (Table 1). Symptoms induced by all of these strains were similar to those produced by a known strain of *X. campestris* pv. *vitians* (strain Sal). Leaves of control plants inoculated with sterile YT medium and carborundum did not develop symptoms.

Two *X. campestris* pv. *vitians* strains were obtained from the Laboratorium voor Microbiologie Gent Culture Collection (LMG 937 and 8689). Strain LMG 937 represented the type A *X. campestris* pv. *vitians* strains that were renamed *X. axonopodis* pv. *vitians*, whereas strain LMG 8689 represented the type B strains that were renamed *X. hortorum* pv. *vitians* (37). Neither strain induced disease symptoms in inoculated lettuce leaves in three independent experiments. In addition, both of these strains were morphologically distinct from the other strains of *X. campestris* pv. *vitians* examined in this study, which were yellow and mucoid. LMG 937 was yellow but nonmucoid, whereas LMG 8689 was white and mucoid.

Fatty acid analysis. Three independent whole-cell fatty acid analyses were conducted with 24 pathogenic *X. campestris* pv.

vitians strains (Table 1). The fatty acid profiles of *S. maltophilia* generated in these three analyses had a mean similarity index of 95% with the profile in the MIS database, indicating the technical accuracy of these analyses (Table 2). The fatty acid profiles of the 24 *X. campestris* pv. *vitians* strains had a mean similarity index of 70% (range of 42 to 88%) with the *X. campestris* profile, consistent with these being strains of *X. campestris*. When the profiles of these strains were compared with those of *X. campestris* pathovars in the database, the closest matches were *X. campestris* pv. *vitians* (54%), *X. campestris* pv. *carotae* (39%), and *X. campestris* pv. *pelargonii* (7%) (Table 2). Moreover, most strains were not consistently identified as a single pathovar, and the pathovar identified as being the closest match varied among replicates (*X. campestris* pv. *vitians* for replicates 1 and 2, and *X. campestris* pv. *carotae* for replicate 3; Table 2). Thus, the results of the fatty acid analyses indicated that the *X. campestris* pv. *vitians* strains were *X. campestris*, but did not consistently identify them as *X. campestris* pv. *vitians* or reveal distinct groups within the strains.

Rep-PCR. The patterns of DNA fragments (hereafter referred to as fingerprints) generated by rep-PCR for all pathogenic strains of *X. campestris* pv. *vitians* were indistinguishable, regardless of the primer used (REP, ERIC, or BOX). In contrast, rep-PCR DNA fingerprints of the nonpathogenic strains LMG 937 and LMG 8689 were different from each other and from the fingerprint of the *X. campestris* pv. *vitians* strains (Fig. 1). The rep-PCR fingerprint of *X. campestris* pv. *vitians* also was distinct from those of the other *X. campestris* pathovars (Fig. 2). These results indicate that the pathogenic *X. campestris* pv. *vitians* strains compose a relatively homogenous group, and that strains LMG 937 and LMG 8689 are genetically distinct.

PCR amplification and sequence analysis of the ITS1 region. The L1/G1 primer pair directs the amplification of the ITS1 sequence from a wide range of bacteria (15). This primer pair directed the amplification of an $\approx$ 600-bp DNA fragment from genomic DNA of 14 strains of *X. campestris* pv. *vitians* (including the nonpathogenic LMG 937 and LMG 8689), 3 strains of *X. campestris* pv. *carotae*, and 2 strains each of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* (Table 1).

TABLE 2. Results of fatty acid analyses of *Xanthomonas campestris* pv. *vitians* strains

Strains	Replicate 1		Replicate 2		Replicate 3	
	Pathovar ^a	Sim. ^b	Pathovar	Sim.	Pathovar	Sim.
Control	<i>S. maltophilia</i>	0.926	<i>S. maltophilia</i>	0.965	<i>S. maltophilia</i>	0.962
Fl-1	<i>vitians</i>	0.552	<i>carotae</i>	0.667	<i>carotae</i>	0.722
9301	<i>vitians</i>	0.843	<i>pelargonii</i>	0.420	<i>carotae</i>	0.654
9302	<i>vitians</i>	0.882	<i>vitians</i>	0.773	<i>carotae</i>	0.788
9303	<i>vitians</i>	0.573	<i>vitians</i>	0.605	<i>carotae</i>	0.654
9304	<i>vitians</i>	0.887	<i>vitians</i>	0.666	<i>pelargonii</i>	0.467
9305	<i>vitians</i>	0.743	<i>vitians</i>	0.766	<i>carotae</i>	0.785
9306	<i>vitians</i>	0.722	<i>vitians</i>	0.654	<i>carotae</i>	0.737
9307	<i>vitians</i>	0.641	<i>vitians</i>	0.830	<i>carotae</i>	0.728
9308	<i>vitians</i>	0.674	<i>carotae</i>	0.574	<i>carotae</i>	0.735
9309	<i>vitians</i>	0.843	<i>vitians</i>	0.809	<i>carotae</i>	0.767
9310	<i>vitians</i>	0.850	<i>carotae</i>	0.740	<i>carotae</i>	0.774
H+	<i>vitians</i>	0.612	<i>carotae</i>	0.745	<i>vitians</i>	0.787
41335	<i>vitians</i>	0.428	<i>vitians</i>	0.789	<i>carotae</i>	0.720
30547	<i>vitians</i>	0.590	<i>carotae</i>	0.693	<i>carotae</i>	0.758
31975	<i>vitians</i>	0.701	<i>carotae</i>	0.717	<i>carotae</i>	0.739
30526	<i>vitians</i>	0.592	<i>carotae</i>	0.743	<i>carotae</i>	0.724
95-02	<i>vitians</i>	0.729	<i>vitians</i>	0.743	<i>carotae</i>	0.761
Pyb251	<i>vitians</i>	0.700	<i>vitians</i>	0.679	<i>carotae</i>	0.789
Sal	<i>carotae</i>	0.487	<i>carotae</i>	0.623	<i>vitians</i>	0.573
KC	<i>vitians</i>	0.666	<i>vitians</i>	0.632	<i>carotae</i>	0.735
GON	<i>vitians</i>	0.664	<i>vitians</i>	0.056	<i>vitians</i>	0.701
9401	<i>vitians</i>	0.869	<i>vitians</i>	0.731	<i>carotae</i>	0.756
B162	<i>vitians</i>	0.863	<i>carotae</i>	0.797	<i>vitians</i>	0.740
LL2	<i>pelargonii</i>	0.593	<i>pelargonii</i>	0.816	<i>pelargonii</i>	0.658

^a *X. campestris* pathovar having the highest matching value for each strain for each replicate.

^b Sim. = similarity indexes (0–1) showing the degree of similarity between the fatty acid profile of the tested strain and the highest matching pathovar identified in the Microbial Identification System database.

There were 554 nucleotide positions aligned as homologous character traits. A BLAST search of the EMBL/GenBank/DBJ database conducted with the sequences of the PCR-amplified fragment from *X. campestris* pv. *vitians* strain Sal revealed a high degree of sequence identity (98 to 100%) with previously determined ITS1 sequences of *X. campestris* pv. *vitians* (strain 700a, AF060176 and strain 701, AF060177). Comparisons then were made with the other *X. campestris* ITS1 sequences determined in this study. The ITS1 sequences of the 14 pathogenic strains of *X. campestris* pv. *vitians* were identical. The ITS1 sequence of *Xyloella fastidiosa* (ATCC 35879) was used as the out-group based upon results of a BLAST search showing that this was the most similar non-*Xanthomonas* sequence (data not shown).

Of these 554 nucleotide positions, 23 were informative. The heuristic search resulted in a singular most parsimonious tree having a length of 29 steps, consistency index of 0.83, a retention index of 0.96 and a rescaled consistency index of 0.80. A cladogram representing this tree is shown in Figure 3. The phylogenetic analysis infers strong support for four distinct distal clades defined on the cladogram as clade A, *Xanthomonas campestris* pv. *vitians* taxa; clade B, tritomy of *X. campestris* pv. *carotae* taxa; clade C, LMG 8689 and LMG 937; and clade D, *X. campestris* pv. *phaseoli* + *X. campestris* pv. *phaseoli* var. *fuscans* taxa. Clade A, consisting of *X. campestris* pv. *vitians* taxa, has an 86% bootstrap and one synapomorphic site, a G > A transition that differentiates it from its sister clade (B) that includes the *X. campestris* pv. *carotae* taxa. The clade that includes clades A + B (*X. campestris* pv. *vitians* + *X. campestris* pv. *carotae* taxa) is supported by a 98% bootstrap and is differentiated completely from clade C by 10 unambiguous synapomorphic sites in their ITS1 nucleotide sequences. This very high number of evolutionary changes in the ITS1 sequences between clade A + B and clade C provides overwhelming evidence of the phylogenetic distance between the *X. campestris* pv. *vitians* taxa examined here and LMG 8689 and LMG 937. This significant genetic difference discovered in this study is consistent with the LMG 8689 and LMG 937 being morphologically distinct from the *X. campestris* pv. *vitians* strains and

their nonpathogenicity to lettuce. Clade D, consisting of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* taxa, is the most basal clade and sister to all remaining *X. campestris* taxa analyzed. One synapomorphy within the ITS1 alignment, a T > C transition, separates the *X. campestris* pv. *phaseoli* taxa from the *X. campestris* pv. *phaseoli* var. *fuscans* taxa. This genetic differentiation is consistent with the latter strains being phenotypically distinct from *X. campestris* pv. *phaseoli* by producing a brown melanin-like pigment in culture. In addition, clade D is supported by an 81% bootstrap, but, more significantly, is differentiated from the remaining *X. campestris* taxa by four synapomorphic sites within the ITS1 alignment.

RFLP analysis. RFLP analysis was performed with 17 strains of *X. campestris* pv. *vitians* from various geographical locations (Table 1). The P2 probe, which contains a repetitive element from the *X. campestris* pv. *phaseoli* genome (13), hybridized to multiple fragments of *X. campestris* pv. *vitians* total genomic DNA digested with *EcoRI* (Fig. 4), *BamHI*, or *HincII*. This indicates that repetitive DNA sequences are present in the *X. campestris* pv. *vitians* genome. No polymorphisms were observed among the strains of *X. campestris* pv. *vitians* when total genomic DNA was digested with *BamHI* (data not shown), whereas polymorphisms were detected when DNA was digested with *EcoRI* (Fig. 4) or *HincII* (data not shown). Strains from the same geographical location often had identical RFLP patterns (e.g., strains 9824, 9844, and 9856 from Santa Maria and Lompoc, CA; strains GON, 9306, and 9401 from Gonzales and Salinas, CA; and strains 9912 and 9916 from Hawaii) (Fig. 4 and data not shown). However, polymorphisms were detected among strains isolated during the same year from the same geographical location (e.g., strains 9862, 9868, and 9873 from Salinas) (Fig. 4). Thus, although strains from a given geographical area tended to have similar RFLP patterns, this was not always the case. This analysis also revealed that these *X. campestris* pv. *vitians* strains are not genetically identical (i.e., clonal).

Detection and analysis of plasmid DNA. Based on the number of bands observed in ethidium bromide-stained agarose gels, seven *X. campestris* pv. *vitians* strains had single plasmids, whereas two strains, B162 (isolated from Guadalupe, CA in 1995) and 31975 (isolated from Dareton, Australia in 1978) had two plasmids (Fig. 5A). When digested with *EcoRI*, *BamHI*, or *SmaI*, distinct banding patterns were detected for the single plasmids from strains Sal, LL2, 41335, 9824, and FI-1, and for the two plasmids from strains B162 and 31975 (Fig. 5B). Similar banding patterns were obtained for plasmid DNAs from the two Hawaiian strains (9916 and 9912; Fig. 5B). Based upon the estimated fragment sizes for digested individual plasmids, the size of these plasmids ranged from 32 to 56 kb.

Fig. 1. Agarose gel showing polymerase chain reaction fingerprint patterns generated from genomic DNA from strains of various *Xanthomonas campestris* pathovars using primers corresponding to enterobacterial repetitive intergenic consensus sequences. Lane M contains DNA size markers (1-kb ladder, Gibco-BRL). Lane 1, strain 9834; lane 2, strain 9844; lane 3, strain 9851; lane 4, strain 9856; lane 5, strain 9862; lane 6, strain 9873; lane 7, strain 9906; lane 8, strain 9912; lane 9, strain 9915; lane 10, strain 9916; lane 11, strain B162; lane 12, strain LL2; lane 13, strain FL-1; lane 14, strain 9303; lane 15, strain 9308; lane 16, strain 41335; lane 17, strain 30526; lane 18, strain LMG 8689; and lane 19, strain LMG 937.

Fig. 2. Agarose gel showing polymerase chain reaction fingerprint patterns generated from genomic DNA from strains of various *Xanthomonas campestris* pathovars using primers corresponding to enterobacterial repetitive intergenic consensus sequences. Lane M contains DNA size markers (1-kb ladder, Gibco-BRL). Lanes 1 and 2, *X. campestris* pv. *vitians* (strains B162 and Sal); lanes 3 and 4, *X. campestris* pv. *carotae* (strains 9936 and 9940); lanes 5 and 6, *X. campestris* pv. *armoraciae* (strains 275 and 756); lanes 7 and 8, *X. campestris* pv. *alfalfae* (strains 101 and 102); lanes 9 and 10, *X. campestris* pv. *campestris* (strains 295 and 404); and lanes 11 and 12, *X. campestris* pv. *phaseoli* var. *fuscans* (strains 54 and 13).

DISCUSSION

The results of the various analyses of genetic diversity of *X. campestris* pv. *vitians* strains associated with recent BLS outbreaks in California and other geographical regions revealed that these strains compose a relatively homogeneous group, rather than being composed of genetically distinct strains. A number of *X. campestris* pathovars are heterogeneous (i.e., composed of genetically diverse strains) including pvs. *begoniae* (40), *citri* (20,40), *dieffenbachiae* (4), *hederae* (24), *mangiferaeindicae* (11), and *vesicatoria* (21,34). These are often pathovars with a relatively wide host range (3,5,40). In most of these cases, the genetically diverse strains within a pathovar were readily identified by various genotypic and phenotypic criteria (5,20,21,24,34,39–41). Based on several of these criteria (e.g., pathogenicity, fatty acid profiles, rep-PCR, and ITS1 sequence analysis), genetically diverse strains were not detected among the pathogenic *X. campestris* pv. *vitians* strains examined in the present study. Thus, these results indicate that *X. campestris* pv. *vitians* is a homogeneous rather than heterogeneous pathovar. This also is con-

Fig. 3. Cladogram showing inferred evolutionary affiliations of 23 strains of *Xanthomonas campestris* pvs. *vitians* (v strains), *carotae* (c strains), *phaseoli* (p strains), and *phaseoli* var. *fuscans* (f strains), and *Xylella fastidiosa* (Xf; ATCC 35879). Cladogram is based on the singular most parsimonious tree resulting from a heuristic search (PAUP) on aligned internal transcribed spacer 1 nucleotide sequences. *X. fastidiosa* served as the outgroup taxon. Branch support is depicted in the cladogram as percent bootstrap support, numbers above branches, and number of unambiguous synapomorphic sites, numbers below branches. Analysis based on 23 informative characters out of 554 aligned characters. Tree statistics: TL, 29 steps; CI, 0.83; RI, 0.96; and RC = 0.80. The LMG 937 strain indicated with an asterisk is the type strain of *Xanthomonas campestris* pv. *vitians*.

Fig. 4. Restriction fragment length polymorphism analysis of *Xanthomonas campestris* pv. *vitians* total genomic DNA digested with *Eco*RI and probed with recombinant plasmid P2, which contains an *X. campestris* pv. *phaseoli* genomic DNA fragment with a repetitive sequence. Lane 1, strain LL2; lane 2, strain B162; lane 3, strain 9862; lane 4, strain 9868; lane 5, strain 9873; lane 6, strain 9916; lane 7, strain 9824; and lane 8, strain 9856.

Fig. 5. Characterization of plasmid DNA extracted from strains of *Xanthomonas campestris* pv. *vitians*. **A**, Agarose gel showing plasmid DNA extracted from *X. campestris* pv. *vitians*. Arrows indicate the smallest (lane 4) and largest (lane 8) plasmids, and C indicates chromosomal DNA. Lane 1, strain Sal; lane 2, strain B162; lane 3, strain LL2; lane 4, strain 41335; lane 5, strain 31975; lane 6, strain 9824; lane 7, strain 9916; lane 8, strain 9912; and lane 9, strain FI-1. **B**, Agarose gel showing plasmid DNA extracted from *X. campestris* pv. *vitians* digested with *Bam*HI. Lanes M contains DNA size markers (Lambda DNA digested with *Hind*III); lane 1, strain Sal (Salinas, CA); lane 2, strain B162 (Guadalupe, CA); lane 3, strain LL2 (Bakersfield, CA); lane 4, strain 41335 (Australia); lane 5, strain 31975 (Dareton, Australia); lane 6, strain 9824 (Santa Maria, CA); lane 7, strain 9916 (Kula, HI); lane 8, strain 9912 (Kula, HI); and lane 9, strain FI-1 (Florida).

sistent with the fact that the host range of *X. campestris* pv. *vitians* is relatively narrow, including leaf and head lettuce types and a number of *Lactuca* spp. such as wild lettuce (*L. serriola* L.) (14,18).

The rep-PCR analysis did not reveal differences among the *X. campestris* pv. *vitians* strains examined in this study, but did allow for differentiation of these strains from other *X. campestris* pathovars. These results provide further evidence that rep-PCR can be used to differentiate pathovars of *X. campestris* (20). For example, Louws et al. (20) found that the rep-PCR fingerprints of *X. campestris* pvs. *campestris*, *phaseoli*, *pelargonii*, and *vesicatoria* were distinct and distinguishable. Furthermore, rep-PCR analysis revealed intraspecific variability among strains of pathovars known to be heterogeneous (e.g., pvs. *vesicatoria*, *citri*, and *campestris*), but not among strains of pathovars considered to be more genetically homogeneous (e.g., pvs. *phaseoli* and *pelargonii*). Thus, the finding that all pathogenic *X. campestris* pv. *vitians* strains had identical rep-PCR fingerprints is consistent with this being a homogeneous pathovar, as opposed to a limitation of the rep-PCR method to reveal genetic diversity within a pathovar. Recently, rep-PCR was used to differentiate strains of *X. campestris* pv. *vitians* from xanthomonad-like bacteria recovered from weeds around lettuce fields in California (1).

Analysis of ribosomal RNA is a well-established tool for revealing relationships among bacteria (42). Because of the high level of sequence conservation that can exist among rRNA genes (16S, 23S, and 5S) at the genus and species levels, the more variable sequences separating these genes (spacer regions) tend to be more useful for differentiating bacteria at these taxonomic levels (15). RFLP analysis of total genomic DNA of various *X. campestris* pathovars with a 16+23S rRNA probe differentiated among pathovars and also revealed variability within certain pathovars (5). RFLP analysis of PCR-amplified *rrs* (16S) and *rrl* (23S) genes did not differentiate various pathovars of *Pseudomonas syringae*, whereas RFLP analysis of the ITS1 spacer region differentiated *P. syringae* pv. *tomato* strains from those of other pathovars (23). The ITS1 rDNA sequence was used recently as a diagnostic tool to help establish that a new disease of leek in California was caused by *P. syringae* pv. *porri* (17). The finding that the ITS1 sequences of the *X. campestris* pv. *vitians* strains were identical, but different from those of other pathovars, was further evidence of the genetic homogeneity of these strains and demonstrates the value of the ITS1 sequence as a diagnostic tool for identification of *X. campestris* pathovars.

The *X. campestris* pv. *vitians* strains examined in this study were not clonal because genetic diversity was detected by the RFLP and plasmid DNA analyses. The RFLP analysis was conducted with a repetitive DNA probe (P2) from *X. campestris* pv. *phaseoli*, which has been used to differentiate strains of *X. campestris* pv. *phaseoli* from *X. campestris* pv. *phaseoli* var. *fuscans*, both of which cause common bacterial blight of bean, and to reveal polymorphisms among strains of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* from different geographical locations (13). Hybridization of the P2 probe with multiple *X. campestris* pv. *vitians* restriction fragments indicates that a related repetitive element is present in the genome. Sequence analysis of the P2 probe has revealed a high level of sequence identity ($\approx 90\%$) to an insertion sequence identified in *X. campestris* pv. *dieffenbachiae* (4; data not shown). Thus, because these repetitive elements are likely to represent mobile genetic elements in genomic and plasmid DNA (7), it is not surprising that hybridization with such an element would reveal some level of diversity within strains of a homogeneous pathovar like *X. campestris* pv. *vitians*. Whereas most genetic diversity was detected among strains from different geographic regions, the finding of genetic diversity among some strains from the same location is consistent with localized evolution or multiple introductions via contaminated seed (2,30).

During the course of this study, we did not find evidence that genetically distinct xanthomonads cause BLS of lettuce. The existence of genetically distinct strains of *X. campestris* pv. *vitians* initially was suggested based upon analyses of protein profiles; however, it is important to note that at least one of the aberrant strains (the pathovar reference strain NCPPB 976=LMG 937) was morphologically distinct and not pathogenic to lettuce (35). Furthermore, it was this strain that was found to be aberrant based on subsequent DNA-DNA hybridization and Biolog GN microplate fingerprint analyses, and used as the basis for proposing two new names for *X. campestris* pv. *vitians*: *X. axonopodis* pv. *vitians* and *X. hortorum* pv. *vitians* (37). Unfortunately, strains of *X. axonopodis* pv. *vitians* and *X. hortorum* pv. *vitians* obtained from Laboratorium voor Microbiologie Gent Culture Collection were not pathogenic to lettuce and were morphologically and genetically distinct from the pathogenic *X. campestris* pv. *vitians* strains used in the present study. Therefore, we were not able to confirm that genetically distinct xanthomonads cause BLS of lettuce. Recently, Schaad et al. (32) rejected the reclassification of *X. campestris* pv. *vitians* type A as *X. axonopodis* pv. *vitians* and *X. campestris* pv. *vitians* type B as *X. hortorum* pv. *vitians*, and recommended that all *X. campestris* strains pathogenic to lettuce be retained as *X. campestris* pv. *vitians* until additional phylogenetic information is available. Our results support those recommendations and also demonstrate the need to confirm the pathogenicity of strains used in phylogenetic and taxonomic studies. On the other hand, it is impossible to exclude the possibility that genetically distinct xanthomonads cause BLS of lettuce.

Crosse (8) and Schroth (33) stressed the importance of knowing the genetic diversity of a pathogen population occurring on a given host in a given locality, and the similarity or differences to other populations occurring on the same host in other localities. They warned of the possible inaccurate representation of an organism as it occurs in nature if studies are confined to only a few strains. The molecular tools used in this study offer a precise and reliable approach for analyzing the genetic diversity of bacterial strains involved with disease outbreaks at a local, regional, or even global scale. In the case of *X. campestris* pv. *vitians*, it seems that a few representative strains could be selected for epidemiological and taxonomic studies, as well for the identification of lettuce cultivars with BLS resistance.

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