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Differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* by PFGE and RFLP

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Abstract

Xanthomonas campestris pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*, the causal agents of the common and fuscous bacterial blight of beans, appear to be phenotypically identical except that the latter can produce a melanin-like pigment in culture. Ten isolates of *X. campestris* pv. *phaseoli* and 12 isolates of *X. campestris* pv. *phaseoli* var. *fuscans* were examined using pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP). The average genome sizes for *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* were 3850.6 ± 48.9 and 3584.3 ± 68.1 kb respectively. The genetic relatedness of the isolates was determined from macrorestriction patterns generated using *Xba*I. Cluster analysis indicated that the non-fuscous and fuscous strains are distinct. RFLP results, based on the highly conserved *hrp* genes and a pectate lyase gene from *Xanthomonas*, also indicated that the two bacteria are genetically different. The results obtained in this study suggest that this pathovar can be segregated into two subgroups under a recently proposed reclassification of the *Xanthomonas* genus.

Introduction

Xanthomonas campestris pv. *phaseoli* and its fuscous variant, *X. campestris* pv. *phaseoli* var. *fuscans* are the causal agents of common bacterial blight of bean (*Phaseolus vulgaris* L.) (Yoshii, 1980). Both pathogens have the same host range and have similar biochemical phenotypes, except that the fuscous variant is able to produce an extracellular brown, melanin-like pigment on tyrosine media due to the secretion and oxidation of homogenistic acid (Goodwin and Sopher, 1994). Homogenistic acid is an intermediate in the tyrosine catabolic pathway. In the non-fuscous strains, a complete catabolic pathway appears to be present which permits utilization of tyrosine as a nutrient (Goodwin and Sopher, 1994). Although both pathogens produce the same symptoms, fuscous and non-fuscous strains may differ in their virulence. In long-term field trials, the fuscous variants were found to be more virulent than non-fuscous isolates (Rudolph, 1990). In addition, a mixed inoculum of fuscous and non-fuscous isolates may cause more severe disease symptoms than either variant alone (Yoshii, 1980). Restriction fragment length polymorphism (RFLP) analysis of genomic and plasmid DNA (Gabriel et al., 1989), DNA–DNA hybridization (Hilderbrand et al., 1990) and amplified DNA polymorphisms (Birch et al., 1997; Xue and Goodwin, 1993) suggest that the fuscous variants may be genetically distinct from the non-fuscous strains.

A pathovar was originally defined by Dye et al. (1980) as 'a strain or a set of strains with the same or similar characters differentiated at infrasubspecific level from other strains of the same species or

subspecies on their distinctive pathogenicity to one or more plant hosts.' Differences in biochemical and serological characteristics between pathovars are not entirely discounted although these characteristics are thought to play a lesser role at the infraspecific level than pathogenicity (Dye et al., 1980). For example, in spite of the difference in melanin-like pigment production, *X. campestris* pv. *phaseoli* and the non-fuscous variant, *X. campestris* pv. *phaseoli* var. *fuscans*, are considered to be synonymous since they infect the same plant hosts (Bradbury, 1984). Moreover, the pathovar classification scheme has no provisions for genetic differences among xanthomonads with the same host range or for the classification of non-pathogenic xanthomonads.

The classification of *Xanthomonas* based on host specificity does not appear to correlate well with the genetic relationships within this genus (Vauterin and Swings, 1997). A reclassification of *Xanthomonas* was proposed by Vauterin et al. (1995). The large *X. campestris* cluster was divided into 16 DNA homology groups, and as a result, *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* were reclassified into the *X. axonopodis* group, which is DNA homology group 9 (Vauterin et al., 1995). However, this group remains the largest and most heterogeneous genomic group, and it has been speculated to contain several subgroups (Vauterin et al., 1995). For the sake of clarity, we have retained the traditional names in this study.

Several studies have suggested that the fuscous and non-fuscous variants are genetically different (Audy et al., 1994; Birch et al., 1997; Gabriel et al., 1989; Lazo and Gabriel, 1987; Lazo et al., 1987; Xue and Goodwin, 1993). However, further evidence is needed. In response to the recently proposed reclassification of *X. campestris* pathovars and the taxonomically ambiguous nature of this pathovar, this study examines the genetic differences within this pathovar by using macrorestriction analysis and pulsed-field gel electrophoresis (PFGE) in conjunction with RFLP analysis. In addition, the chromosomal sizes of a number of *X. campestris* pathovars were determined.

Materials and methods

Bacterial strains and media

Strains used in this study are listed in Table 1. Bacterial strains were routinely maintained on YDC agar (Schaad and Stall, 1988). Stock cultures were stored in peptone–yeast–glycerol broth (NYGB) (Turner et al., 1984) containing 30% (v/v) glycerol at −80 °C.

Preparation of bacterial DNA

Bacterial cells were grown in TY broth (Beringer, 1974) overnight at 30 °C on a rotary shaker (300 rpm). Cells were embedded in 1% (w/v) SeaKem GTG agarose (FMC Corporation) as previously described by Cooksey and Graham (1989) with the following modifications. Bacterial cells were mixed with the agarose at 37 °C, and the mixtures were dispensed into plastic molds (10 mm by 5 mm by 1.5 mm) and placed on ice. To facilitate cell lysis, each agarose block was cut into two pieces prior to lysis, and the pieces were placed in a 1.5 mL microcentrifugation tube containing 0.5 mL of lysis solution. The tubes were incubated at 56 °C overnight. Agarose blocks were subsequently washed and stored as per Cooksey and Graham (1989).

Restriction enzyme digestion and pulsed-field gel electrophoresis

The restriction endonuclease *Xba*I (Pharmacia LKB Biotechnology) was chosen for this study. Two agarose blocks from each bacterial strain were incubated at 37 °C for 1 h in 500 µL of 1× restriction buffer prior to digestion. Each digestion was carried out in a final volume of 200 µL containing the appropriate restriction buffer and 20–30 units of restriction enzyme. After overnight incubation at 37 °C, the reaction was stopped by two 1 mL washes with TE buffer (pH 8.0) (Sambrook et al., 1989).

PFGE was performed using a contour-clamped homogeneous electric field apparatus (Pulsaphor™ System; Pharmacia LKB Biotechnology) (Chu et al., 1986). Restriction fragments were separated in 15 by 15 cm 1% (w/v) agarose gels (Boehringer Mannheim) in 0.5× Tris–borate–EDTA buffer (TBE) (Sambrook et al., 1989). An electrophoresis buffer of 0.5× TBE and a buffer temperature of 16 °C were used throughout the study. Restriction fragments between 10 and 150 kb were resolved using 0.3–18 s switching pulse over a ramp period of 32 h at 8 V cm^{-1}. Restriction fragments greater than 150 kb were separated by 3–35 s switching pulses over a ramp period of 42 h at 8 V cm^{-1}. Size standards were λ concatemers (Bio-Rad Laboratories) and λ DNA cut with *Hin*dIII (Pharmacia LKB Biotechnology). Following electrophoresis, each

Table 1. Source and geographical origin of bacterial strains used in this study

Strains	Geographical origin	Source
X. campestris pv. *phaseoli*		
90-6	Ontario, Canada	This lab
b957	Brazil	R. Aline
BXP18	Ontario, Canada	B.N. Dhanvantari
BXP98	Ontario, Canada	B.N. Dhanvantari
G24	Kansas, USA	J.L. Leech
G27†	Nebraska, USA	A. Vidaver
G47	Nebraska, USA	R. Shuster
G62	Florida, USA	R.E. Stall
G68	Missouri, USA	J. Fletcher
X50	Columbia	R. Gilbertson
X. campestris pv. *phaseoli* var. *fuscans*		
90-1	Ontario, Canada	This study
BXPF12	Ontario, Canada	B.N. Dhanvantari
BXPF65	Ontario, Canada	B.N. Dhanvantari
BXPF118	Ontario, Canada	B.N. Dhanvantari
Empola duro	Brazil	R. Gilbertson
G46	Nebraska, USA	R. Shuster
M1	Malawi	R. Gilbertson
M6	Malawi	R. Gilbertson
M9	Malawi	R. Gilbertson
M10	Malawi	R. Gilbertson
XcpF310	Ontario, Canada	B.N. Dhanvantari
Xcf Wallen	Alberta, Canada	L.W. Kaychuk
X. campestris pv. *alfalfae*		
b974	Idaho, USA	R. Aline
X. campestris pv. *cucurbitae*		
ATCC 23378	Idaho, USA	R. Aline
X. campestris pv. *campestris*		
90-50	Arkansas, USA	W. Wiebe
A4-81-1-1	California, USA	W. Wiebe
PHW117	Louisiana, USA	W. Wiebe
X. campestris pv. *vesicatoria*		
87-14D	North Carolina, USA	W. Wiebe
P437-4	Taiwan	W. Wiebe

† Type strain ATCC 49119.

gel was stained in an ethidium bromide solution and photographed with transmitted UV light at 295 nm.

Restriction fragment length polymorphism

RFLP analysis was conducted using two cloned DNA fragments from *X. campestris* as probes. The cosmid pIJ3225 from *X. campestris* pv. *campestris* contains a region of approximately 25 kb of *hrp* genes (Arlat et al., 1991). The plasmid pPL-1 contains a 1.4 kb pectate lyase gene from *Xanthomonas campestris* pv. *vesicatoria* (Beaulieu et al., 1991).

PFGE gels were blotted onto nylon membranes (Micron Separations Inc.) using standard methods. Probes were labeled with [α-^{32}P] dCTP (ICN) using the random-priming technique according to the manufacturer's instruction (Boehringer Mannheim). Hybridization with the labeled probes was performed at 65 °C. High stringency washing conditions were conducted throughout the RFLP experiments according to standard methods (Sambrook et al., 1989). The blots were subsequently exposed to X-ray films (X-Omat; Eastman Kodak Company) at −80 °C with an intensifying screen.

Image and statistical analyses

The photographic negatives and prints were digitally scanned using an AS-1 Array Scanner (Array Technologies, Inc.) and were subsequently manipulated using the Bio Image Electrophoresis Analyzer (Millipore Inc.). The digitized images were compared to photographic prints for measurements of restriction fragment migration distances and comparisons of restriction patterns between strains.

Dissimilarity coefficients (Cooksey and Graham, 1989; Denny et al., 1988) between all strains were calculated based on their restriction patterns. A dissimilarity coefficient matrix was prepared and analyzed by cluster analysis with MacDendro 1.01 (Thioulouse, 1989) using the unweighted pair-group method with averages (UPGMA) (Sneath and Sokal, 1973). Results of the cluster analysis were subsequently used to construct a dendrogram by GraphMu 4.11 (Thioulouse, 1989).

Results

PFGE and macrorestriction analysis

Digestion of chromosomal DNA from *Xanthomonas* strains with *Xba*I consistently generated 28–51 distinct restriction fragments depending on the bacterial strains (Figures 1 and 2). Variations were not observed between bacterial preparations of agarose embedded DNA or between separate PFGE gels, and three or more independent experiments were conducted on individual strains. The largest and smallest restriction fragments observed after digestion with *Xba*I were 397.9 ± 1.4 kb for *X. campestris* pv. *phaseoli* strain G62, and 4.6 ± 0.1 kb for *X. campestris* pv. *phaseoli* strain X50. The wide range of fragment sizes required two separate sets of switching pulses times in order to resolve the restriction fragments.

A dendrogram was constructed using cluster analysis based on pairwise comparisons of the macrorestriction polymorphism. The dendrogram yielded three distinct clusters designated as cluster A, B and C (Figure 3). The *X. campestris* pv. *phaseoli* isolates were grouped into cluster A. All fuscous isolates are in cluster B but are separated into two subgroups by isolates of other pathovars such as *X. campestris* pv. *alfalfae, X. campestris* pv. *cucurbitae,* and *X. campestris* pv. *vesicatoria*. *X. campestris* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* can be separated into two distinct groups at a similarity coefficient of 0.26 suggesting that the genetic unrelatedness of strains in the *phaseoli* pathovar is directly associated with the ability of the pathogens to produce the extracellular brown pigment. Moreover, the macrorestriction results showed that the *fuscans* strains were more closely related to the other pathovars tested than to the non-fuscous strains. The heterogeneous cluster B is segregated from cluster C, consisting of only *X. campestris* pv. *campestris* strains, at a similarity coefficient of 0.32.

Although a number of isolates formed small clusters based on their geographical origin, a clear segregation based on origin cannot be concluded. For example, the *X. campestris* pv. *phaseoli* var. *fuscans* isolates M1, M6, M9 and M10 from Malawi were segregated into two clusters, but these clusters had a low similarity (0.41) to each other (Figure 3). Similarly, *X. campestris* pv. *phaseoli* var. *fuscans* isolates 90-1, BXPF118 and BXPF12 from Ontario formed a smaller subgroup, whereas two other Ontario isolates, BXPF65 and XcpF310, are segregated from this subgroup. Therefore, no clear clustering of the isolates can be made according to geographical origin.

RFLP analyses

Distinctive hybridization patterns for the fuscous and non-fuscous *X. campestris* pv. *phaseoli* groups were produced by the plasmid probe pPL-1 and the cosmid probe pIJ3225 (Figures 4 and 5). RFLP results also clearly demonstrated that co-migrating fragments are genetically related. There was little or no sizable polymorphism between strains within the fuscous and non-fuscous groups of *X. campestris* pv. *phaseoli* for the two probes tested. An exception was where the cosmid probe pIJ3225 hybridized to the 212.6 ± 1.7 kb band in all of the fuscous strains with the exception of *X.campestris* pv. *phaseoli* var. *fuscans* Xcf Wallen (Figure 5). For strain Xcf Wallen, pIJ3225 hybridized to a 328.1 ± 2.7 kb *Xba*I restriction fragment. The *Xba*I restriction profile of *X. campestris* pv. *phaseoli* var. *fuscans* Xcf Wallen shows that this strain lacks a 212.6 kb fragment (Figure 1).

With the exception of *X. campestris* pv. *campestris* and *X. campestris* pv. *cucurbitae*, the other pathovars tested in cluster B produced hybridization patterns similar to the fuscous strains. The lack of polymorphism between these pathovars is in good agreement with the results obtained from the PFGE analysis. Although both probes showed homology to a restricted

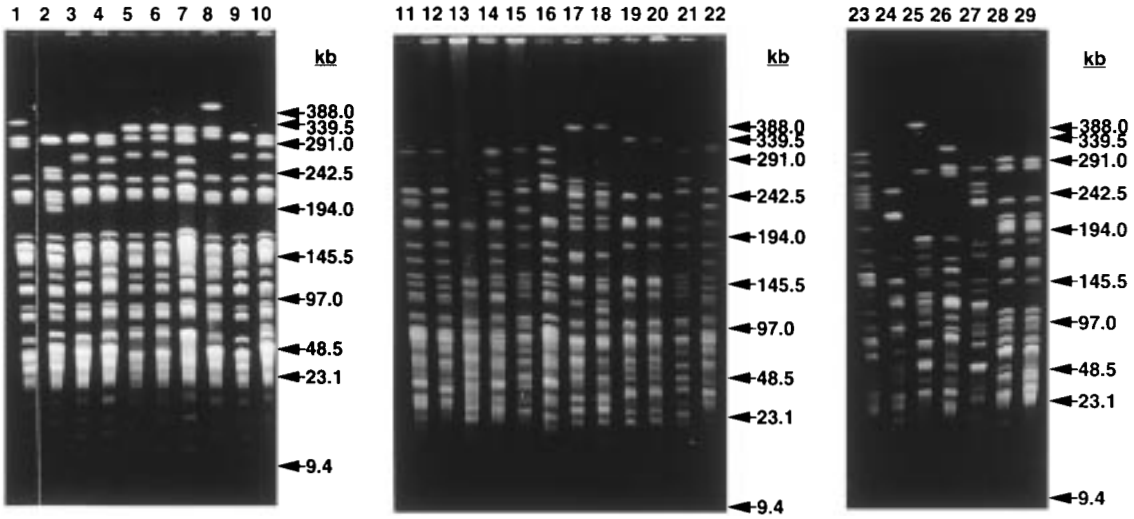

Figure 1. PFGE gel of strains of *X. campestris* digested with *Xba*I. The PFGE conditions were 1% (w/v) agarose gel in 0.5× TBE, switching pulses of 3–35 s over a ramp period of 42 h at 8 V cm^{-1}. Lanes 1–10 contained *X. campestris* pv. *phaseoli* strains 90-6, b957, BXP18, BXP98, G24, G27, G47, G62, G68, and X50. Lanes 11–22 contained *X. campestris* pv. *phaseoli* var. *fuscans* strains 90-1, BXPF12, BXPF65, BXPF118, Empola duro, G46, M1, M6, M9, M10, F310 and Xcf Wallen. Lane 23 contained *X. campestris* pv. *alfalfae* strain b974. Lane 24 contained *X. campestris* pv. *cucurbitae* strain ATCC 23378. Lanes 25–27 contained *X. campestris* pv. *campestris* strains 90-50, A4-81-1-1 and PHW117. Lanes 28 and 29 contained *X. campestris* pv. *vesicatoria* strains 87-14D and P437-4.

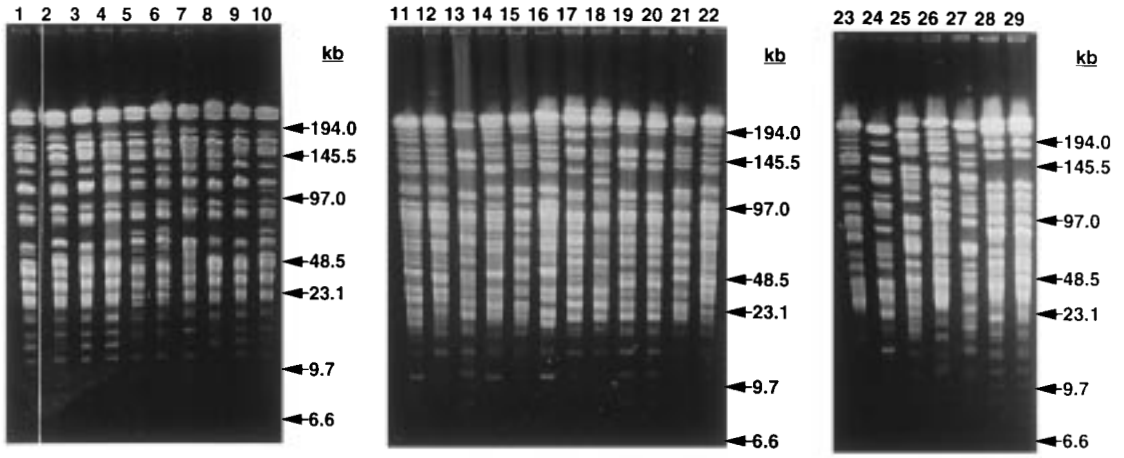

Figure 2. PFGE gel of strains of *X. campestris* digested with *Xba*I. The PFGE conditions were 1% (w/v) agarose gel in 0.5× TBE, switching pulses of 0.3–18 s over a ramp period of 32 h at 8 V cm^{-1} (for details of samples in the lanes refer to legend for Figure 1).

fragment in all of the strains tested, there is a weak homology between *X. campestris* pv. *cucurbitae* ATCC 23378 and the plasmid probe pPL-1 (Figure 4). Similarly, the cosmid probe pIJ3225 and strain ATCC 23378 have little homology (Figure 5). There is a degree of polymorphism observed in the *X. campestris* pv. *campestris* cluster C when either the pPL-1 or pIJ3225 probe is used.

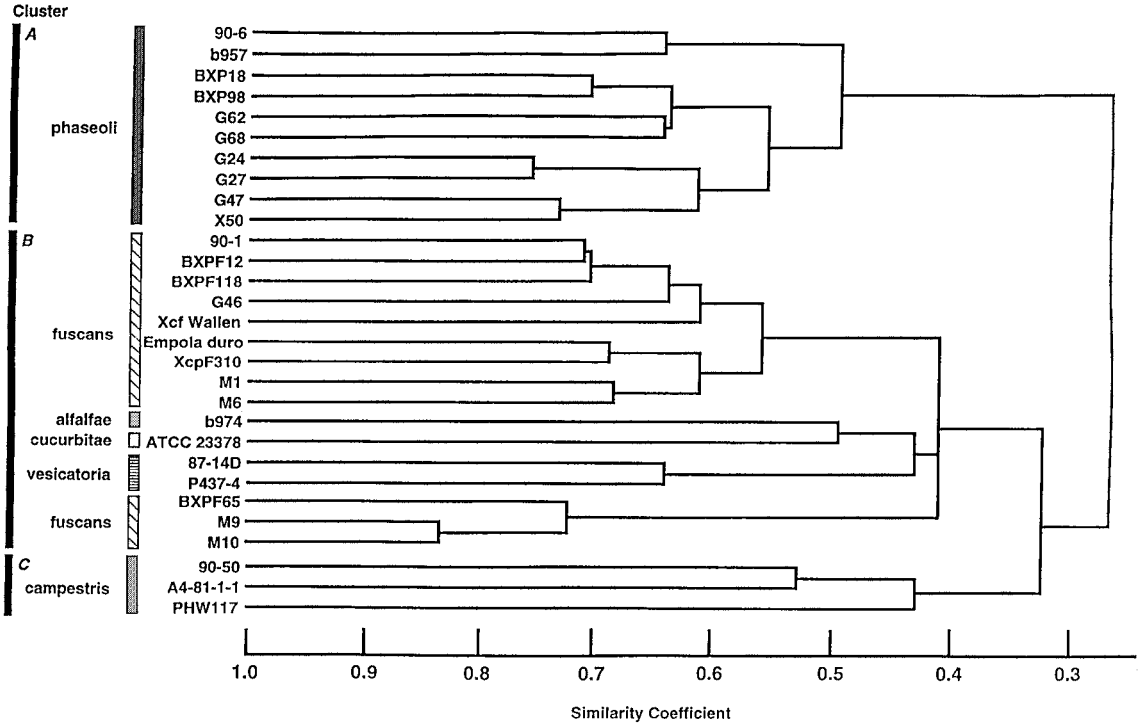

Figure 3. Dendrogram of strains of *X. campestris* based on cluster analysis of dissimilarity coefficients determined from *Xba*I macrorestriction patterns.

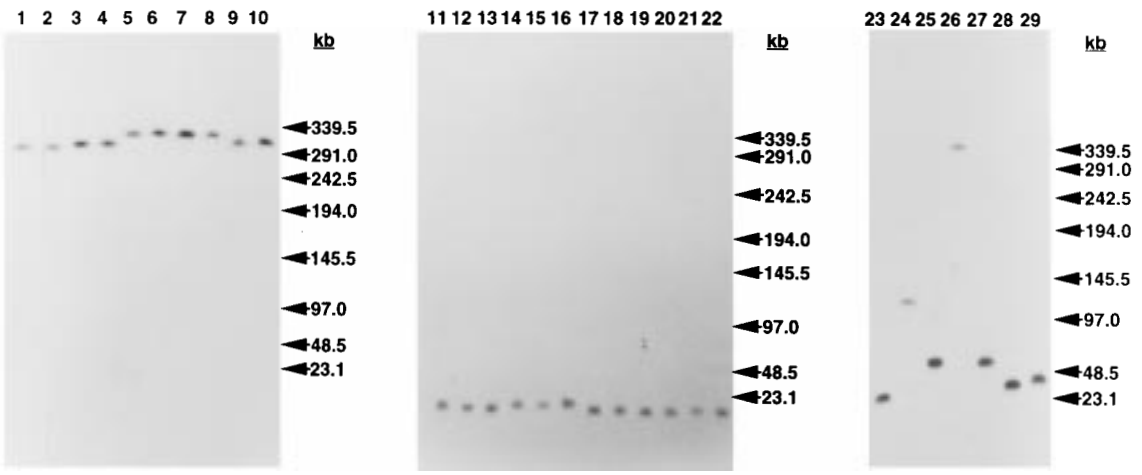

Figure 4. RFLP analysis using the plasmid probe pPL-1 (pectate lyase) under high stringency conditions. *X. campestris* strains were restricted with *Xba*I and subjected to PFGE conditions as outlined in Materials and methods (for details of samples in the lanes refer to legend for Figure 1).

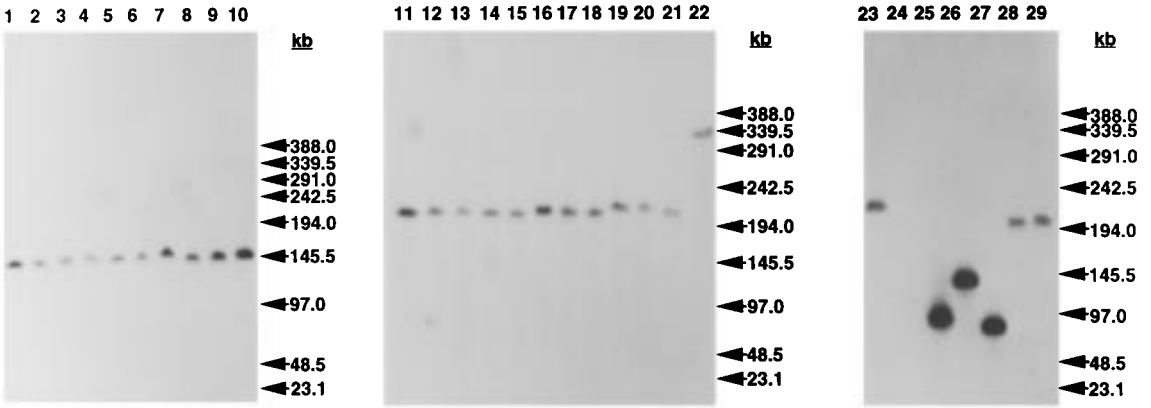

Figure 5. RFLP analysis using the cosmid probe pIJ3225 (pathogenicity and hypersensitivity response) under high stringency conditions. *X. campestris* strains were restricted with *Xba*I and subjected to PFGE conditions as outlined in Materials and methods (for details of samples in the lanes refer to legend for Figure 1).

Similar to the results obtained with PFGE, RFLP analysis suggests that isolates from bean cannot be distinguished according to their geographical origin.

Chromosome size

The mean chromosome size of each strain was determined from the total value of the *Xba*I generated fragments. Since two parameters were used to separate the restriction fragments, fragment sizes larger than 150 kb (Figure 1) that were separated by the 3–35 s switching pulses were measured and added to fragment sizes smaller than 150 kb (Figure 2) that were separated by the 0.3–18 s switching pulses. The chromosome sizes of the *X. campestris* strains are shown in Table 2. Among all strains characterized in this study, *X. campestris* pv. *cucurbitae* strain ATCC 23378 had the smallest genome size (2567.6 ± 12.8 kb) while *X. campestris* pv. *phaseoli* strain G47 had the largest genome size (4353.9 ± 17.9 kb). The difference between the average chromosome sizes for *X. campestris* pv. *phaseoli* and its fuscous variant was approximately 266 kb.

Discussion

Characterizing the genetic relationship of *X. campestris* pathovars by examining polymorphism among restriction fragments generated with frequent-cutting restriction endonucleases, such as *Eco*RI, and resolving the fragments by conventional agarose gel electrophoresis has been difficult due to the large number of restriction fragments produced (Lazo and Gabriel, 1987). To circumvent these problems, PFGE can be used to separate large restriction fragments generated by rare-cutting restriction endonucleases (Schwartz and Cantor, 1984; Smith and Condemine, 1990).

PFGE and rare-cutting restriction endonucleases have been used to distinguish genomic variation among species and pathovars of the genus *Xanthomonas* (Cooksey and Graham, 1989; Davis et al., 1997; Graham and Chase, 1992). However, these studies did not include *X. campestris* pv. *phaseoli var. fuscans* nor did they report on the chromosome sizes of any *X. campestris* pathovars.

The molecular weight of the genome of *X. campestris* pv. *campestris* strain B-24 was previously determined to be 2.20×10^9 using initial renaturation kinetics (Kamper et al., 1985) and is equivalent to a chromosome size of 3390 kb (assuming 649 Da per base pair). In order to reduce the problem of size underestimation due to considerable co-migration of restriction fragments (Lucier and Brubaker, 1992), our study used two separate sets of pulsing parameters to resolve the restricted fragments. The size of the chromosomes obtained from six different pathovars in Table 2 are consistent with the size estimated from the renaturation kinetics. The consistency of our results from at least three independent experiments suggests that the measured chromosome sizes for all of the pathovars are highly reproducible using PFGE and *Xba*I digestion.

Table 2. Genome sizes of various strains of *X. campestris* calculated from *Xba*I-generated restriction fragments

Strains	Genome size ± SE (kb)‡
X. campestris pv. *phaseoli*	
90-6	3581.3 ± 15.3
b957	3610.5 ± 25.4
BXP18	3432.9 ± 12.6
BXP98	4147.3 ± 14.4
G24	3889.8 ± 14.6
G27†	3912.1 ± 10.1
G47	4353.9 ± 17.9
G62	3985.4 ± 10.5
G68	3714.3 ± 23.4
X50	3878.8 ± 18.9
Average	3850.6 ± 48.9
X. campestris pv. *phaseoli* var. *fuscans*	
90-1	3913.2 ± 73.7
BXPF12	3551.8 ± 61.0
BXPF65	3938.2 ± 67.5
BXPF118	3694.9 ± 50.0
Empola duro	3659.3 ± 43.1
G46	4318.1 ± 41.6
M1	3906.2 ± 67.2
M6	3724.2 ± 22.6
M9	2919.4 ± 23.7
M10	2824.1 ± 17.5
XcpF310	3346.9 ± 24.8
Xcf Wallen	3695.4 ± 22.1
Average	3634.3 ± 68.1
X. campestris pv. *alfalfae*	
b974	4181.2 ± 32.7
X. campestris pv. *cucurbitae*	
ATCC 23378	2567.6 ± 12.8
X. campestris pv. *campestris*	
90-50	3089.1 ± 14.9
A4-81-1-1	3042.5 ± 8.9
PHW 117	2907.6 ± 15.4
Average	3013.1 ± 28.0
X. campestris pv. *vesicatoria*	
87-14D	3435.4 ± 20.8
p437-4	3797.6 ± 29.9
Average	3616.5 ± 82.6

†Type strain ATCC 49119.

‡Mean of at least three independent experiments ± standard error (SE).

The classification scheme of *Xanthomonas* has been the subject of intense scrutiny using a polyphasic approach (Vauterin et al., 1995, 1997). Some studies were able to demonstrate that certain pathovars are heterogeneous and have reclassified a number of pathovars into new classification schemes, while studies on the genetic relatedness of other pathovars, such as *X. campestris* pv. *phaseoli*, have remained inconclusive (Birch et al., 1997; Gabriel et al., 1988; Young et al., 1991).

Strains of *X. campestris* pv. *phaseoli* and the fuscous variant can be grouped into a single cluster based on their SDS-PAGE protein patterns and can be further subdivided into two smaller subclusters, which are

unrelated to extracellular brown pigment production (Vauterin et al., 1991). In addition, a number of fuscous strains cannot be clustered based on their electrophoretic protein patterns, and a few strains from pathovars such as pv. *alfalfae* and pv. *cucurbitae* were shown to belong in the two subclusters (Vauterin et al., 1991). After testing 189 strains of *X. campestris* pathovars for 295 phenotypic features, most of the *X. campestris* pathovars were found to be phenotypically indistinguishable except for pathogenicity (Van den Mooter and Swings,1990). These studies suggest that *X. campestris* pv. *phaseoli* cannot be clearly distinguished from other *X. campestris* pathovars by their phenotypes or protein profiles, but only by the pathovar concept of Dye et al. (1980).

Although some studies contend that both pv. *phaseoli* and pv. *phaseoli* var. *fuscans* belong to the same DNA–DNA homology group (Vauterin et al., 1990, 1995), there is evidence to suggest that fuscous strains are genetically distinct from non-fuscous strains of *X. campestris* pv. *phaseoli* by virtue of low DNA–DNA homology in S1 DNA–DNA hybridization studies (Hilderbrand et al., 1990). Despite the low DNA–DNA homology between the fuscous and non-fuscous strains, both belonged to a loose cluster of strains composed of pathovars pathogenic to plants in the Fabaceae family. However, further examination of 24 xanthomonads representing 23 different *X. campestris* pathovars and *X. fragariae* showed that pathogenicity towards members of the same plant host family was not an absolute indicator of genomic relatedness (Hilderbrand et al., 1990).

Differences in the plasmid DNA of the two variants have also been characterized. A 2.0 kb *Eco*RI plasmid fragment from *X. campestris* pv. *phaseoli* was able to hybridize to plasmids isolated from *X. campestris* pv. *phaseoli* but not to plasmids from *X. campestris* pv. *phaseoli* var. *fuscans* and other pathovars (Lazo and Gabriel, 1987). Polymorphisms were found that distinguished *X. campestris* pv. *phaseoli* from the fuscous variant strains when a 3.4 kb *Eco*RI plasmid fragment probe was used (Gilbertson et al., 1989). Furthermore, a set of PCR primers derived from the sequence of the 3.4 kb *Eco*RI fragment was able to produce an additional group of PCR products only in the fuscous strains under certain amplification conditions (Audy et al., 1994). Birch et al. (1997) also distinguished fuscous from non-fuscous strains using a random primer that resulted in a single PCR product in fuscous strains but multiple bands of varying sizes in non-fuscous strains. Similarly, *X. campestris* pv. *phaseoli* could be distinguished from the fuscous variant utilizing chromosomal RFLPs with probes derived from *X. campestris* pv. *citri* (Lazo and Gabriel, 1987). These studies suggest that the two variants can be distinguished by genetic differences despite the lack of any significant differences in host range or symptoms.

Gabriel et al. (1989) previously proposed that *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* be reclassified as *X. phaseoli* and *X. campestris* pv. *fuscans*, respectively (Gabriel et al., 1989; Lazo et al., 1987). However, RFLP analysis with a limited number of probes may lack the robustness and accuracy required to serve as an adequate tool for bacterial taxonomy (Vauterin et al., 1990), and as a result, the proposal of Gabriel et al. (1989) to reinstate *X. phaseoli* based strictly on RFLP analyses was found to be inadequate (Young et al., 1991).

In this study, we avoided underrepresenting *X. campestris* pv. *phaseoli* by examining a geographically diverse collection of the bean pathogen. In contrast, only a limited number of isolates from a few other pathovars were included. The other pathovars were included solely to aid in the comparison of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*, and no firm conclusions are intended to be drawn about the genetic variation of these other pathovars from this study. However, for *X. campestris* pv. *phaseoli* and the fuscous isolates, the macrorestriction and RFLP analyses clearly indicate that they are in separate clonal groups. This conclusion is directly supported by a similar clustering of many of the same isolates of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* used in this study by using amplified DNA polymorphism with primers based on the sequences of a putative two-component signal transduction system of *X. campestris* pv. *campestris* (Xue and Goodwin, 1993) and RFLP analyses using a cloned fragment of a two-component signal transduction system of *X. campestris* pv. *phaseoli* var. *fuscans* (Chan et al., 1998).

The clustering of all of the *X. campestris* pv. *phaseoli* isolates was independent of their geographical locations suggesting that either the dissemination of these pathogens occurred frequently or was a relatively recent event. This is not surprising since the primary mode of transmission of this pathogen has been the worldwide distribution of contaminated seeds (Saettler, 1991). On the basis of RFLP analysis, a lack of clustering of *X. campestris* pv. *phaseoli* isolates obtained from

various regions of the United States was also reported (Gabriel et al., 1989).

Division of certain pathovars into separate taxonomic groups has been reported for other pathovars. For example, *X. campestris* pv. *citri* originally consisted of five subgroups. However, subsequent results clearly suggested that the pathovar is composed of three clonal groups as opposed to the original five (Gabriel et al., 1988, 1989; Hartung and Civerolo, 1987; Vauterin et al., 1995). All three clonal groups were reclassified into genomic group 9 and were named *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *aurantifolia* and *X. axonopodis* pv. *citrumelo* (Vauterin et al., 1995).

Another example is *X. campestris* pv. *vesicatoria* groups A and B, which have traditionally been classified under the same pathovar. However, experimental results based on a diverse number of biochemical, serological and molecular techniques indicated that groups A and B have different genetic and biochemical backgrounds despite similar host ranges (Bouzar et al., 1994; Canteros et al., 1995; Stall et al., 1994; Vauterin et al., 1995). Vauterin et al. (1995) reclassified *X. campestris* pv. *vesicatoria* group A as *X. axonopodis* pv. *vesicatoria* in genomic group 9, and group B as *X. vesicatoria* in genomic group 14.

Two probes, the *hrp* gene cluster and a pectate lyase gene, were used in our RFLP analysis. The *hrp* cluster is required for both pathogenicity and hypersensitivity response (Arlat et al., 1991). In addition, the *hrp* genes from *X. campestris* pv. *vesicatoria* were shown to be highly conserved in many pathogenic xanthomonads (Bonas et al., 1991; Leite et al., 1994). However, the single *X. campestris* pv. *cucurbitae* isolate, unlike isolates from other pathovars in our study, has low homology to the *hrp* gene cluster isolated from *X. campestris* pv. *campestris*. Under the reclassification scheme of Vauterin et al. (1995), *X. campestris* pv. *cucurbitae* belongs to genomic group 8. The results obtained in this and other studies (Bonas et al., 1991; Leite et al., 1994; Stall and Minsavage, 1989) strongly suggest that a large number of *Xanthomonas* pathovars should be tested prior to using the *hrp* genes for comprehensive diagnostic purposes in plant material.

The pectate lyase gene is highly conserved in a number of pectolytic and non-pectolytic xanthomonad isolates (Beaulieu et al., 1991). Although pectolytic activity in our collection of fuscous and non-fuscous strains of *X. campestris* pv. *phaseoli* was not detected (data not shown), all isolates displayed homology to the pectate lyase gene.

The clustering of *X. campestris* pv. *alfalfae*, *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *phaseoli* isolates based on the macrorestriction and RFLP analyses in this study support the reclassification proposal of Vauterin et al. (1995). The results also showed that *X. campestris* pv. *phaseoli* var. *fuscans* is clonally distinct from *X. campestris* pv. *phaseoli*, and is more related to other pathovars in genomic group 9. This study suggests that the genomic group 9 can be further subdivided into smaller clonal groups.

Two scenarios can also be hypothesized from this study. The results suggest that either *X. campestris* pv. *phaseoli and X. campestris* pv. *phaseoli* var. *fuscans* were derived from two separate evolutionary lines through convergence, or that the two clonal groups were derived from a common evolutionary line through divergence. The results from this and other studiesd can neither support nor dispute either hypothesis. Nevertheless, *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans* can clearly be recognized as distinct clonal groups. The new nomenclature given by Vauterin et al. (1995) for these isolates are *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. The evidence presented in this paper and other studies suggest that the fuscous variants may be more appropriately considered as separate subspecies within genomic group 9.

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