Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions

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The taxonomy and evolutionary relationships among members of the genus *Xanthomonas* associated with tomato and pepper have been a matter of considerable controversy since their original description in 1921. These bacteria, which are a major affliction of tomato and pepper crops in warm and humid regions, were originally described as a single species, but subsequent research has shown the existence of at least two genetic groups differentiated by physiological, biochemical and pathological characteristics. This work synthesizes the findings from several approaches, including pathogenicity tests, enzymic activity, restriction fragment analysis of the entire genome, DNA–DNA hybridization and RNA sequence comparisons based on a 2097 base sequence comprising the 16S rRNA gene, the intergenic spacer located between the 16S and 23S rRNA genes and a small region of the 23S rRNA gene. Within the group of xanthomonads pathogenic on pepper and tomato four distinct phenotypic groups exist, of which three form distinct genomic species. These include *Xanthomonas axonopodis* pv. *vesicatoria* (A and C group), *Xanthomonas vesicatoria* (B group) and *Xanthomonas gardneri* (D group). On the basis of phenotypic and genotypic differences between A- and C-group strains, the C strains should be considered as a subspecies within *Xanthomonas axonopodis* pv. *vesicatoria*.

Keywords: fluorimetric DNA hybridization, pulsed field gel electrophoresis, bacterial taxonomy, APIZYM, phylogenetic analysis

INTRODUCTION

Bacterial spot disease on pepper and tomato is a major problem where high temperatures and high humidity occur simultaneously. Ethel M. Doidge (Doidge, 1921) originally identified the causal agent in South Africa as *Bacterium vesicatorium*. In the same year, Gardner and Kendrick (1921) identified a similar organism in the United States, naming it *Bacterium exitiosum*. When they became aware of Doidge's work, they deferred to her taxonomy and adopted *B. vesicatorium* as the

name of the causal agent (Gardner & Kendrick, 1921). However, they noted one difference between the two strains: the bacterium isolated by Doidge was feebly amyolytic, whereas their strains were strongly amyolytic. Two years later, Gardner & Kendrick (1923) identified bacterial spot disease on pepper, determined that it was the same affliction observed on tomato and classified it as *B. vesicatorium*. In subsequent nomenclatural revisions, the bacterium was transferred to *Xanthomonas* (Dowson, 1939) as *Xanthomonas vesicatoria* and eventually to *Xanthomonas campestris* pv. *vesicatoria* (*X. c. vesicatoria*) by Dye (1978). Although considerable phenotypic diversity within the organism was observed in the 1960s (Dye *et al.*, 1964) it was not until the 1990s that it was known that more than one species was responsible for the *Xanthomonas* diseases on pepper and tomato. Stall *et al.* (1994) and Vauterin *et al.* (1990) determined that two genetically distinct groups existed within *X. c. vesicatoria*. Stall *et*

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The GenBank/EMBL/DBJ accession numbers for the 2097 base fragment of strains XV1111¹, XV153, XV338, XV938, *Xanthomonas campestris* pv. *campestris* (XCC¹) and '*Xanthomonas gardneri*' (XCGA2) are, respectively: AF123088, AF123089, AF123090, AF123091, AF123092 and AF123093.

al. (1994) designated the two groups as A and B. Recently, Vauterin *et al.* (1995) reclassified the xanthomonads and divided those in *X. c. vesicatoria* into two species, *X. vesicatoria* and *Xanthomonas axonopodis* pv. *vesicatoria* (*X. a. vesicatoria*). On the basis of the studies by Stall *et al.* (1994) and the reclassification scheme of the *Xanthomonas* genus presented by Vauterin *et al.* (1995), the group A strains are now members of *X. a.* pv. *vesicatoria* and the group B strains are members of *X. vesicatoria*.

Bouzar *et al.* (1994a) documented phenotypic diversity in a worldwide collection of strains which represented the two new species. The strains were variable in terms of carbon substrate utilization patterns, serovar and fatty acid profiles. One phenotypic trait that proved useful for differentiating the two species was starch hydrolysis (Bouzar *et al.*, 1996); 252 strains were compared and it was demonstrated that the ability to significantly hydrolyse starch in less than 2 d was associated only with *X. vesicatoria* (B group) strains and not with *X. a. vesicatoria* (A group) strains. Later, analysis of strains from the Caribbean and Central America revealed that considerable phenotypic diversity existed in Central America (Bouzar *et al.*, 1999, 1994b). Strains were identified which were not typical of A- or B-group strains. Furthermore, several amyolytic strains, which were isolated from samples collected in Mexico (Bouzar *et al.*, 1996) and the Midwest of the United States, differed from typical B strains by reacting with a panel of monoclonal antibodies like A strains (Bouzar *et al.*, 1994a, b, c, 1996): these are designated group A1 in this paper. Another group of amyolytic strains was discovered (Bouzar *et al.*, 1996; Jones *et al.*, 1995), but these strains differed from the B-group strains both serologically and pathogenically and were designated group C (Bouzar *et al.*, 1996).

In 1957, a bacterial disease of tomato was identified (Šutic, 1957) and originally classified as '*Pseudomonas gardneri*'. Later, Dye (1966) examined '*P. gardneri*' and determined that it was a typical xanthomonad according to morphological and biochemical tests. Dye (1966) concluded that '*Xanthomonas gardneri*' was a synonym of *X. vesicatoria*, since both bacteria produced a similar disease on tomato and could not be distinguished by physiological and pathological procedures. Placement of '*P. gardneri*' in the genus *Xanthomonas* was further supported by DNA-rRNA hybridization studies (De Ley, 1978). However, in DNA-DNA hybridization tests '*X. gardneri*' and *X. c.* pv. *vesicatoria* had very low reassociation values with each other, but *X. gardneri* showed reassociation of more than 60% with two other pathovars (Hildebrand *et al.*, 1990; Palleroni *et al.*, 1993). We presented preliminary information showing that several *Xanthomonas* species are pathogenic on tomato, including '*X. gardneri*', *X. vesicatoria* (group B) and *X. a.* pv. *vesicatoria* (group A) (Bouzar *et al.*, 1995).

In recent reports, DNA sequence comparisons have provided a new window into evolutionary relation-

ships among prokaryotes (Olsen *et al.*, 1994). In this paper we present phenotypic and genotypic information on the diversity of *Xanthomonas* strains associated with tomato and/or pepper, demonstrate that at least three distinct evolutionary lineages exist and clarify the relationships among '*X. gardneri*', *X. vesicatoria* and *X. a. vesicatoria*.

METHODS

Bacterial strains and storage. Seventeen *Xanthomonas* strains isolated from tomato and pepper and representing distinct phenotypic and genotypic groups were selected for characterization. The origin of the strains is described in Table 1 and their phenotypic traits are documented in Table 2. Other strains used in this study included the type strains of *Xanthomonas albilineans*, *X. axonopodis*, *X. campestris*, *Xanthomonas fragariae* and *Xanthomonas oryzae*. The strains were stored in 15% glycerol at -70°C for long-term preservation.

mAb production and serological typing of strains. A representative group C strain, XV938, was used as the immunogen to produce a mAb. The mAb was produced as previously described by Bouzar *et al.* (1994a). ELISA reactions were converted to positive and negative reactions with a cut-off for positive reactions at $A_{405} \geq 0.1$.

Pathogenicity tests. Single colonies were transferred to nutrient broth and shaken overnight at 30°C and the bacterial suspensions were centrifuged. The resulting pellets were resuspended in sterile tap water and the suspensions of cells were standardized to an optical density at 600 nm of 0.3 (approx. 5×10^8 c.f.u. ml $^{-1}$). Seedlings of pepper (cultivar Early Calwonder) and tomato (cultivar Bonny Best) were grown in a greenhouse in 15-cm pots containing a steamed peat/vermiculite mixture; a soluble 20:20:20 fertilizer (0.4 g per pot; Peters Fertilizer Products, W. R. Grace) was added to the plants biweekly. To observe the disease symptoms produced by the different strains, leaves of both tomato and pepper plants were inoculated by swabbing inoculum onto leaves that were not fully expanded, by using a cotton applicator saturated with a bacterial suspension amended with carborundum. After inoculation, symptom development was observed periodically for 3 weeks.

Enzymic activity. We used the API gallery, type API ZYM (API BioMérieux) to compare 19 enzymic reactions of the strains listed in Table 1. Bacterial growth was suspended in sterile saline and adjusted to $1.5\text{--}1.8 \times 10^9$ c.f.u. ml $^{-1}$. Enzymic activity was determined according to the manufacturer's recommendations. The tests were scored on a scale from 0 to 5. Comparative data for each strain-enzymic reaction was tabulated and the similarity coefficient between two strains was calculated with the SIMINT program from NTSYS-pc version 1.80 (Exeter Software). Cluster analysis by the unweighted pair-group method was performed using the SAHN program from NTSYS-pc. Data for amyolytic and pectolytic activities were obtained from a previous study (Bouzar *et al.*, 1994a).

Restriction endonuclease analysis. Genomic DNAs of the bacterial strains (Table 1) were prepared and digested with restriction endonucleases *Xba*I, *Vsp*I and *Spe*I as described by Egel *et al.* (1991). The resulting large DNA fragments were separated by PFGE. The gels were stained with ethidium bromide and photographed with type 55 Polaroid

Table 1. *Xanthomonas* strains

See Table 2 for characteristics of groups A, A1, B, C and D.

Current nomenclature	Strain	Original designation	Source*
<i>X. albilineans</i>	XAL ^T	ATCC 12785 ^T	ATCC
<i>X. axonopodis</i> pv. <i>axonopodis</i>	XAX ^T	ATCC 19312 ^T	ATCC
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV153	75-3	Stall
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV155	85-16	Stall
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV157	89-10	Hibberd
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV330	Barbados 1	Jones
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV338	Barbados 2	Jones
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV1085	Mexico 1	Jones
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV1605	Ohio	Miller
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV938	Florida	Jones
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV1220	Thailand	Jones
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	XV1484	Mexico 2	Jones
<i>X. campestris</i> pv. <i>campestris</i>	XCC ^T	ATCC 33913 ^T	ATCC
<i>X. campestris</i>	XV1194	XCV857	Louws
<i>X. fragariae</i>	XF ^T	ATCC 33239 ^T	ATCC
' <i>X. gardneri</i> '	XCGA2	XG101	Hildebrand
' <i>X. gardneri</i> '	XV444	Costa Rica 1	Jones
' <i>X. gardneri</i> '	XV451	Costa Rica 2	Jones
' <i>X. graminis</i> '	XG	ATCC 29091	ATCC
<i>X. oryzae</i>	XOA ^T	ATCC 35933 ^T	ATCC
<i>X. vesicatoria</i>	XV1111 ^T	ATCC 35937 ^T	ATCC
<i>X. vesicatoria</i>	XV56	X525-85	Nagai
<i>X. vesicatoria</i>	XV141	71-4	Starr
<i>X. vesicatoria</i>	XV144	BV20-3A	Alippi

* Details of sources: ATCC, American Type Culture Collection; A. Alippi, Universidad Nacional de la Plata, La Plata, Buenos Aires, Argentina; A. Hibberd, Redlands Research Centre, Cleveland, Queensland, Australia; J. B. Jones, University of Florida, Gulf Coast Research and Education Center, Bradenton, FL, USA; F. Louws, Michigan State University, Plant Research Laboratory, Department of Energy, Plant Biology Building, East Lansing, MI, USA; H. Nagai, Instituto Agronomico, Sao Paulo, Brazil; S. A. Miller, Ohio State University, Plant Pathology Department, Wooster, OH 44691, USA; R. E. Stall, University of Florida, Department of Plant Pathology, Gainesville, FL, USA; M. P. Starr, University of California at Davis, Davis, CA, USA.

Table 2. Phenotypic groups of phytopathogenic xanthomonads associated with tomato and pepper

Phenotypic group	Strain	Distribution	mAb*	Protein (kDa)	PFGE†	Amylolytic activity‡	Pectate hydrolysis
A	XV153, XV155, XV157	Worldwide	1, 21	32	A	—	—
A1	XV1085, XV1605, XV330, XV338	Barbados, Mexico, Ohio	1, 21	25	A	+	+
B	XV56, XV144, XV141, XV1111 ^T	Worldwide	8, 15	27	B	+	+
C	XV938, XV1220, XV1484	Mexico, Thailand, USA	30	27	C	+	+
D	XCGA2, XV444, XV451	Costa Rica, Yugoslavia	8	27	D	—	—

* mAb developed using *Xanthomonas campestris* pv. *vesicatoria* strains that react in ELISA (Bouzar *et al.*, 1994b).

† PFGE group (see Fig. 2). Group A includes A and A1 strains from Fig. 2.

‡ +, Starch hydrolysis visible in less than 2 d.

film. All strains were run on the same gel and similarity coefficients were determined for strains by comparing fragments greater than 100 kb (Egel *et al.*, 1991). Similarity values were calculated as described by Egel *et al.* (1991) by using the mathematical equation proposed by Nei and Li (1987) based on the proportion of shared DNA fragments. The number of nucleotide substitutions per site was estimated by the iterative method of Nei (1987) by using the SAS program as described by Leite *et al.* (1994). The KITSCH program from the PHYLIP computer package (Felsenstein, 1995) was used to infer a rooted phylogenetic tree by using the Fitch–Margoliash method (Fitch & Margoliash, 1967). The input data consisted of a matrix of pairwise genetic distances determined as estimates of the numbers of nucleotide substitutions per site on the basis of similarity coefficients calculated for the combined digestion data of the three enzymes (Leite *et al.*, 1994).

Sequence determination and phylogenetic analysis. Isolation of total genomic DNA, PCR and electrophoresis conditions were as described elsewhere (Ausubel *et al.*, 1987). Amplification of *rrs* (the 16S rRNA gene) and the intergenic spacer (IGS) located between *rrs* and *rrl* (i.e. the 23S rRNA gene) was performed with standard PCR conditions (Innes *et al.*, 1995) using primers FGPS6-63 (5'-GGA GAG TTA GAT CTT GGC TCA G-3') and FGPL132'-38 (5'-CCG GGT TTC CCC ATT CGG-3') as previously described (Ponsonnet & Nesme, 1994).

Six additional oligo primers were synthesized at the DNA Synthesis Core Laboratory (University of Florida, Gainesville, FL, USA) for sequencing of the PCR product. These primers were HAB1 (5'-CCG CAC CTT CCG ATA CG-3'), HAB2 (5'-GTG CGT AGG TGG TGG TTT-3'), HAB3 (5'-CCC AGG CGG CGA ACT T-3'), HAB4 (5'-AAC CGA CTG CTT TTC TTT-3'), HAB5 (5'-AAG CCA ATC CCA GAA ACC-3') and HAB6 (5'-ATC CCG ACA GGC TCC AC-3').

PCR products were purified with Ultrafree MC 30000 MW filters (Millipore). Double-stranded sequencing reactions were conducted at the University of Florida DNA Sequencing Core Laboratory using ABI Prism Dye Terminator cycle sequencing protocols (part number 402078) developed by Applied Biosystems (Perkin-Elmer). Oligo primers (i.e. HAB1–HAB6) were designed using OLIGO 5.0 (National BioSciences) and synthesized at the University of Florida DNA Synthesis Core Laboratory. The fluorescently labelled extension products were analysed with an automated DNA sequencer (model 377; Applied Biosystems). Raw data from the sequencer were edited and aligned using SEQUENCHER version 3.0 (Gene Codes) and the default options in the program CLUSTAL W (Thompson *et al.*, 1994). All samples were sequenced in the forward and reverse directions to ensure the accuracy of nucleotide sequence designations.

Sequence divergences between haplotypes, including the gaps, were estimated with the Kimura two-parameter model (Kimura, 1980) with a 3:1 transition:transversion ratio, using the algorithm in PHYLIP version 3.57 (Felsenstein, 1993). To elucidate relationships among haplotypes, evolutionary trees were generated with the neighbour-joining method (Saitou & Nei, 1987) in MEGA (version 1.02; Kumar *et al.*, 1994) and the parsimony approach of PAUP version 3.1.1 (Swofford, 1993). Support for nodes in both the neighbour-joining and the parsimony approaches was

assessed with a bootstrap confidence level using 500 replicates.

DNA–DNA hybridization. DNA–DNA relatedness analysis was performed in a microplate and determined using the fluorimetric assay of Ezaki *et al.* (1989) with minor modifications. Large molecular mass DNA was extracted from strains of *X. vesicatoria* and *X. axonopodis* pv. *vesicatoria* representative of the RFLP groups identified in this study and the type strains of *X. c. campestris*, *X. albilineans*, *X. fragariae*, *X. oryzae*, *X. axonopodis* pv. *axonopodis* (*X. a. axonopodis*) and '*Xanthomonas graminis*'. DNA extraction and purification were performed according to Marmur's procedure, as described by Johnson (1994). The DNA was fragmented by three passages through a French pressure cell under 110316 kPa, resulting in DNA fragments approx. 0.5 kb in size. The DNA was heat-denatured and used either for coating microdilution plates (MicroFluor type B; Dynatech) or biotinylated for use as a probe. Each microtitre well was coated with 3 µg fragmented, denatured DNA. The probe contained 20–50 ng DNA labelled with Photoprobe biotin (Vector) according to the manufacturer's protocol. Hybridization was carried out at 52 °C for 12 h. DNA reassociation ratios were determined fluorimetrically (7630 Microplate Fluorometer; Cambridge Technology) 1 h after the binding of β -galactosidase avidin D (Vector Laboratories) and the addition of the 4-methylumbelliferyl- β -D-galactoside substrate (Sigma). Hybridization experiments were repeated three times and consisted of three replications of each probe with every strain.

RESULTS

mAb reactions

mAb 30 was produced and tested against several hundred strains representing A- and B-group strains, representative strains of other *Xanthomonas* species, more than 100 group C strains and all strains used in this study. It reacted with all group C strains, but not with any of the other strains including those shown in Table 2.

Pathogenicity tests

All strains (with the exception of XV1194) used in this study that were isolated from pepper or tomato were pathogenic on pepper, tomato or both (Table 3).

Table 3. Pathogenic reaction of selected strains isolated from tomato and pepper on 'Early Calwonder' pepper and 'Bonny Best' tomato

Strain	Disease reaction	
	Pepper	Tomato
XCC ^T , XV1194	–	–
XV153, XV155, XV157, XV1085, XV1605, XV330, XV338, XV444, XV451	+	+
XV938, XV1220, XV1484, XV1111 ^T , XV56, XV141, XV144, XCGA2	–	+

+, Disease; –, hypersensitive reaction.

Fig. 1. Clustering of group A, B, C and D strains based on enzymic activity using the API ZYM gallery.

Although XV1194 was isolated from tomato, symptoms typical of a disease reaction were not observed on either pepper or tomato. However, a reaction characteristic of hypersensitivity was observed on those plants 5 d after inoculation. On cabbage seedlings, XV1194 induced leaf spots and stem lesions similar to those induced by *Xanthomonas campestris* pv. *armoraciae* and *Xanthomonas campestris* pv. *raphani*; infection did not result in typical black-rot symptoms typical of *X. c. campestris*.

Enzymic activity analysis

A dendrogram was constructed using the clustering method of NTSYS. Two major clusters were evident (Fig. 1). One large cluster consisted of strains from groups A, A1 and C. A second but more divergent cluster consisted of two B strains (XV56 and XV141), the ‘*X. gardneri*’ strain (XCGA2) originally from Yugoslavia and two D strains (XV444, XV451) from Costa Rica. Considerable variability existed between strains within the B group, the *X. vesicatoria* type strain (XV1111^T) being very remote from the two other B strains (XV56 and XV141). *X. a. axonopodis* (XAX^T), *X. c. campestris* (XCC^T) and XV1194 were distantly related to xanthomonads pathogenic on tomato and/or pepper.

Restriction endonuclease analysis

In the dendrogram generated from the similarity coefficient obtained by averaging the similarity coefficients for *Spe*I, *Xba*I and *Vsp*I, five clusters were

Fig. 2. Clustering of group A, B, C and D strains inferred from similarity coefficients obtained from *Spe*I, *Xba*I and *Vsp*I restriction endonuclease data. The tree was generated by the KITSCH procedure with the PHYLIP computer package by using the Fitch–Margoliash method. The genetic distances are estimates of the numbers of nucleotide substitutions per site.

distinguished (Fig. 2). Greater genetic divergence was observed for strains in the A group than for strains in any of the other groups. The A strains formed two

Fig. 3. Phylogenetic relationships among six strains of *Xanthomonas*, based on 2097 bases of 16S rRNA and the adjacent intergenic sequence. A parsimony analysis using PAUP yielded a single most parsimonious tree that was exactly concordant with a neighbour-joining tree. The consistency index (CI), the retention index (RI) and bootstrap values for nodes supported above 50% are based on the parsimony analysis. The distance scale given above the tree (percentage sequence divergence along single branches) is based on the neighbour-joining analysis.

clusters. One cluster contained typical A strains (XV153, XV155 and XV157) and two of the amylolytic A1 strains (XV1085 and XV1605), whereas the other cluster consisted of two amylolytic A1 strains (XV330 and XV338) from Barbados. The B strains formed a separate, less divergent cluster than the A group. The three C-group strains formed a very tight cluster sharing a high percentage of similarity and were most distantly related to the other groups. Strains representative of the D group (XV444 and XV451) clustered with the '*X. gardneri*' (XCGA2) strain. Strain XV1194 and, to a lesser extent, the type strain of *X. c. campestris* did not cluster with any group.

RNA sequence comparisons

A fragment of 2097 bases was resolved in all six strains. The sequence data have been submitted to GenBank. Overall diversity was low, with 39 variable sites containing 22 transitions, 7 transversions and 11 single-base indels. Site 1985 contained both an indel and a transversion.

Genetic distances between lineages ranged from $d = 0.0014$ (between group A strain XV153 and group A1 strain XV338) to $d = 0.0118$ (between strain XV153 and group B strain XV1111^T). All tree-building methods resolved two clusters of haplotypes: strains XV1111^T (group B), '*X. gardneri*' (group D) and *X. c. campestris* (XCC^T) were distinguished from the group A strains XV153 and XV338 and from the group C strain XV938 with a mean divergence of $d = 0.0105$. Seventeen steps in the parsimony analysis distinguished these two clusters. The highest divergence within either cluster was $d = 0.0033$, or nine steps in the parsimony analysis.

One most parsimonious tree generated by the PAUP method shows 100% support for a split into two monophyletic groups: XV1111^T, '*X. gardneri*' (XCGA2) and *X. c. campestris* (XCC^T) forming one of the groups and XV153, XV338 and XV938 forming the other (Fig. 3). A dendrogram generated by the

neighbour-joining method shows the same basic topology, including the two primary lineages observed in the parsimony analysis. Bootstrap support for the primary split in this phylogeny was 100%. Support for the branching order within either cluster was 52–64% in the neighbour-joining analysis and 59–65% in the parsimony analysis. This low level of support is presumably due to the shallow divergence between strains XV153 and XV338 (three site substitutions) and between strains XV1111^T and '*X. gardneri*' (five site substitutions).

DNA–DNA hybridization

When the representative A strain, XV153, was used as the probe, the levels of hybridization with group A strains and the group C strain was higher than 80%, whereas the value was lower than 50% against groups B and D (including '*X. gardneri*') strains, *X. a. axonopodis* (XAX^T) and the other *Xanthomonas* spp. (Table 4). When the two amylolytic A1 strains (XV330 and XV1085) were used as probes, relationships were observed which were similar to those that occurred with XV153. When the representative group C strain, XV938, was used as the probe, the level of hybridization was greater than 60% with the A1 strains (XV1085 and XV1605), between 50 and 60% with the A strains (XV153 and XV155) and less than 50% with all other strains tested. When the type strain of *X. vesicatoria* (XV1111^T) was used as the probe, the level of hybridization with XV56, another B-group strain, was greater than 80%, whereas the level of hybridization with strains from other groups was less than 50%. The levels of hybridization between XV444, a group D strain and '*X. gardneri*' (XCGA2) was greater than 90% when each strain was used as a probe, whereas with DNA from other strains hybridization was less than 40%. When XV1194 was used as a probe, it hybridized weakly with all strains except the type strain of *X. c. campestris*. The same was true for *X. c. campestris* when it was used as a probe.

Table 4. Comparison of percentage DNA reassociation values for 17 *Xanthomonas* strains

Values are means of six readings and are normalized to the hybridization value of each probe.

Probe	Phenotypic group	1	2	3	4	5	6	7	8	9	10	11	12	13	XAL	XOA	XG	XF
1. XV153	A	100	101	100	90	91	82	28	35	37	37	32	40	47	15	37	20	42
2. XV155	A	95	100	90	90	82	72	30	28	33	35	32	36	—	—	—	—	—
3. XV1085	A1	77	81	100	100	84	81	30	30	41	40	38	37	35	14	34	21	40
4. XV1605	A1	83	80	96	100	86	68	31	33	38	37	33	45	—	—	—	—	—
5. XV338	A1	74	75	84	89	100	78	28	32	35	34	30	36	43	14	34	20	37
6. XV938	C	53	55	77	71	65	100	32	31	37	34	34	36	39	14	34	19	44
7. XV56	B	30	31	36	37	37	35	100	86	44	35	36	45	—	—	—	—	—
8. XV1111 ^T	B	29	31	38	36	35	40	83	100	44	40	37	44	26	12	21	16	28
9. XV444	D	26	27	30	33	29	32	31	32	100	91	34	40	—	—	—	—	—
10. XCGA2	D	31	26	37	31	33	34	28	29	98	100	34	42	33	12	32	20	37
11. XV1194	—	31	31	37	37	35	39	32	41	42	41	100	93	—	—	—	—	—
12. XCC ^T	—	26	29	35	30	31	30	30	35	32	38	73	100	24	16	28	20	35
13. XAX ^T	—	50	—	54	—	52	56	—	32	—	38	—	31	100	13	43	18	40

DISCUSSION

The phenotypically distinct *Xanthomonas* strains associated with bacterial spot of tomato and pepper collected from diverse locations (Bouzar *et al.*, 1994a, b; Stall *et al.*, 1994) were determined to be distinct genotypically. Although two previous studies (Stall *et al.*, 1994; Vauterin *et al.*, 1995) showed that strains within the previously accepted specific epithet of *X. c. pv. vesicatoria* were actually two distinct species, we present further evidence to show that more than two distinct species cause disease on tomato and pepper.

Previously, strains associated with the species *X. c. vesicatoria* were shown (Stall *et al.*, 1994; Vauterin *et al.*, 1995) to form two genetic groups (i.e. A and B). The two groups were readily differentiated from each other in that B strains strongly hydrolysed starch and, to a lesser extent, degraded pectate, whereas A strains hydrolysed starch only after 14 d and were unable to degrade pectate (Bouzar *et al.*, 1996; Stall *et al.*, 1994). However, amylolytic and pectolytic strains typical of the B group were recently collected in the United States, Barbados and Mexico. Many of these strains were shown to be related to strains in the A group, on the basis of reaction profiles with mAbs, whereas others were different from both group A and B (Bouzar *et al.*, 1999). On the basis of the unique phenotypic characteristics of these strains they were placed in two new phenotypic groups. The first phenotypic group, group A1, had PFGE patterns and serological reactions similar to those of A-group strains. DNA–DNA hybridization data confirmed the close relationship between A1 and A strains. Strains from the second group of amylolytic strains were phenotypically and genotypically distinct from A and B strains and were designated as group C. This group of strains can easily be distinguished from A and A1 strains on the basis of PFGE profiles and reactions to antibodies and bacteriophages (J. B. Jones, H. Bouzar & P. D. Roberts, unpublished results). However, on the basis of phylogenetic data, group C strains are closely related to those of groups A and A1. DNA–DNA hybridization data indicate that group C strains are

distinct from group A strains, but that the two groups are close enough to be included in the same species as group A strains. Clearly, there are significant phenotypic and genotypic differences between A- and C-group strains, and the C strains should be considered as subspecies within *X. a. vesicatoria*.

The phylogenetic tree obtained from the sequence analysis of the 16S rRNA gene and the IGS located between *rrs* and *rrl* clearly produced two clades on the tree. One branch included the representative B-group strain, '*X. gardneri*' and *X. c. campestris*, whereas the other included the representatives of groups A, A1 and C. The DNA–DNA hybridization data corroborate the 16S rRNA sequencing data. The C strains, although phenotypically distinct from the A and B groups, were more closely related to the A strains. When Hauben *et al.* (1997) looked at sequence analysis of the 16S rRNA, only slight differences were detected between many xanthomonads. However, much of the variation needed to show differences between closely related species appears to be associated with the IGS, as shown by Leblond-Bourget *et al.* (1996). Furthermore, although close identity based on sequence analysis of 16S rRNA may indicate that two organisms may be members of the same species, it is not necessarily the case, as demonstrated with *Bacillus* spp. in which 16S rRNA sequences were extremely close but DNA–DNA homologies were low (Fox *et al.*, 1992). The converse has not been shown, i.e. where members of a species have high DNA–DNA homologies but low rRNA sequence similarity. Coupled with the fairly high relatedness between A and C strains based on DNA–DNA hybridization, the 16S rRNA sequence data appear to support the close genetic relationship between these two groups (Bouzar *et al.*, 1995).

Šutic (1957) identified a tomato disease in Yugoslavia caused by '*P. gardneri*' in 1957. This organism was considered to be a synonym of '*X. vesicatoria*' (Dye, 1966) and this status was maintained for years. However, Hildebrand *et al.* (1990) and Palleroni *et al.* (1993), using DNA–DNA hybridization tests, deter-

mined that '*X. gardneri*' was not closely related to the B group (*X. vesicatoria*), but was more closely related to several other pathovars of *X. campestris*. Comparisons were not made in either study with typical A-group (*X. a. vesicatoria*) strains. We demonstrated in this study that '*X. gardneri*' is not closely related to either group A or group B but is instead a member of a third species that is pathogenic on tomato and/or pepper. Furthermore, '*X. gardneri*' appears to occur rarely in nature, the type strain being the only known strain in existence. Analysis of an extensive collection of strains (Bouzar *et al.*, 1994a) did not reveal any '*X. gardneri*' strains; however, in a survey of *Xanthomonas* strains collected in the Caribbean and Central America, an organism very similar to '*X. gardneri*' was found in Costa Rica (Bouzar *et al.*, 1999, 1994b). On the basis of PFGE analysis, two of these strains (i.e. XV444 and XV451) collected in Costa Rica appeared to be closely related phylogenetically to '*X. gardneri*'. Furthermore, DNA-DNA hybridization confirmed that these strains from Costa Rica and '*X. gardneri*' belong to the same species.

From our analyses, it is evident that '*X. gardneri*' strains are pathogens of tomato and/or pepper and that they are phenotypically and genetically distinct from both *Xanthomonas exitiosa* (*X. vesicatoria sensu* Vauterin *et al.*) and *X. vesicatoria* (*X. a. vesicatoria sensu* Vauterin *et al.*). Furthermore, they represent a species that is distinct from the latter two. Further comparisons with the type strains of the other *Xanthomonas* species will be needed in order to place them in the correct species.

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