Genotypic characterization of xanthomonad strains isolated from passion fruit plants (*Passiflora* spp.) and their relatedness to different *Xanthomonas* species

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The genetic diversity of 55 xanthomonad strains isolated from passion fruit plants (*Passiflora* spp.) and identified as *Xanthomonas campestris* pv. *passiflorae* was initially assessed by randomly amplified polymorphic DNA (RAPD) analysis. The strains showed a high level of polymorphism with almost unique fingerprints. Fifteen clusters with a similarity of ~ 70% were identified, three of which were prevalent. There was a correlation between the clusters and the geographic origin of the strains. A representative strain of each cluster, together with the pathovar reference strain, were used to verify the relationships of these strains to 18 *Xanthomonas* species and *Pseudomonas syringae* pv. *passiflorae*. All *Xanthomonas* species yielded a unique RAPD profile and no consistent relatedness to the *X. campestris* pv. *passiflorae* strains was observed. Amplification products were also analysed by repetitive (rep) primers (BOX, ERIC and REP), RFLP of the 16S–23S rDNA intergenic spacer and SDS-PAGE of whole-cell proteins. All of these approaches generated profiles characteristic for each *Xanthomonas* species but the taxonomic position of the *X. campestris* pv. *passiflorae* strains could not be unequivocally assigned. Finally, DNA–DNA hybridization allowed a sound taxonomic allocation of the strains to *Xanthomonas axonopodis* pv. *passiflorae*.

Keywords: *Xanthomonas*, RAPD, rep-PCR, RFLP-16S–23S, SDS-PAGE of whole-cell proteins

INTRODUCTION

Xanthomonas campestris is a species complex including mainly phytopathogens which cause diseases in various economically important plants. The species includes pathovars designated according to their hosts. *X. campestris* pv. *passiflorae* (Pereira, 1969) Dye 1978, a pathogenic bacterium of passion fruit (*Passiflora* spp.), is a pathovar rarely included in general or taxonomic studies, perhaps because the host plant is cultivated in tropical and subtropical regions. In recent years passion fruit has become a commercial product in the Brazilian juice industry and large-scale cultivation now occurs. Only two varieties of passion fruit

are exploited commercially (*Passiflora edulis* Sims and *Passiflora edulis* f. *flavicarpa*), although about 400 species have been described (Souza & Meletti, 1997). An area of 25000 ha is currently used to produce passion fruit in Brazil (Teixeira, 1994) and the commercial demand has been increasing gradually. Concomitant with the expansion of passion fruit production, the incidence of disease is also rising (Torres Filho & Ponte, 1994). Bacteriosis, caused by *X. campestris* pv. *passiflorae*, is a major disease limitation in the development of cultivated areas in Brazil. The disease, designated premature death, has been increasing since the late 1970s (Torres Filho & Ponte, 1994). Infection occurs through natural openings and lesions and results in systemic invasion of the whole plant. In the leaves, the disease causes necrosis and soaked, greasy lesions of irregular shape surrounded by chlorotic areas. The fruit is also affected, with the

Abbreviations: IGS, intergenic spacer; RAPD, randomly amplified polymorphic DNA.

Table 1. *X. campestris* pv. *passiflorae* strains used

Strains	Host	Sampling site	State*
8930, 11061, 11323, 11324, 11325, 11327, 11329, 11330, 11331	<i>Passiflora edulis</i>	Londrina	PR
11341, 11342	<i>Passiflora edulis</i>	S. Sebastião da Amoreira	PR
11043	<i>Passiflora edulis</i>	Siqueira Campos	PR
11343	<i>Passiflora edulis</i>	Arapoti	PR
11405	<i>Passiflora edulis</i>	Marumbi	PR
11406	<i>Passiflora edulis</i>	Apucarana	PR
11407	<i>Passiflora edulis</i>	Nova Esperança	PR
120, 428, 444, 445	<i>Passiflora edulis</i>	Campinas	SP
121, 148	<i>Passiflora edulis</i>	Valinhos	SP
617	<i>Passiflora edulis</i>	Registro	SP
658	<i>Passiflora alata</i>	Guaíçara	SP
777	<i>Passiflora edulis</i>	Bragança Paulista	SP
941, 960, 1007	<i>Passiflora edulis</i>	Vera Cruz	SP
996, 997	<i>Passiflora edulis</i>	Tupã	SP
1039	<i>Passiflora edulis</i>	Pilar do Sul	SP
1040	<i>Passiflora edulis</i>	Paulínia	SP
1151	<i>Passiflora edulis</i>	S. João da Boa Vista	SP
1154	<i>Passiflora edulis</i>	Jaguariúna	SP
1166	<i>Passiflora edulis</i>	Dracena	SP
1168	<i>Passiflora edulis</i>	Cosmópolis	SP
1171	<i>Passiflora alata</i>	Indaiatuba	SP
674	<i>Passiflora</i> sp.	Araguari	MG
649, 714, 715, 712, 717, 767, 1053, 621	<i>Passiflora</i> sp.	Brasília	DF
686, 687, 688	<i>Passiflora</i> sp.	Feira de Santana	BA
720, 748	<i>Passiflora</i> sp.	Propriá	SE
591, 592, 593	<i>Passiflora</i> sp.	Ibiapaba	CE
ICMP 3151†	<i>Passiflora</i> sp.	Araraquara	SP

* State in Brazil; see legend to Fig. 1.

† Pathovar reference strain from the International Collection of Microorganisms from Plants, Auckland, New Zealand.

appearance of hard greasy spots making it unsuitable for consumption and industrial processing.

Reclassification of the genus *Xanthomonas* has been proposed by Vauterin *et al.* (1995) based mainly on DNA–DNA hybridization analysis, although the phytopathological identification was retained. Accordingly, the species *X. campestris* was divided into 16 new species and the Sub-Committee on Taxonomy of Plant-pathogenic Bacteria has validated this proposal (see Young *et al.*, 1996). However, xanthomonads from passion fruit plants have not been analysed and the former designation, *X. campestris* pv. *passiflorae*, has been maintained.

Genotypic diversity is being analysed increasingly by PCR-based methods. By using short arbitrary primers [randomly amplified polymorphic DNA (RAPD)-PCR] or primers binding to repetitive extragenic sequences, BOX, ERIC and REP, generally known as rep, high-resolution genomic fingerprinting can be obtained (Welsh & McClelland, 1990; Versalovic *et al.*, 1991). The rep primers correspond to conserved

motifs in bacterial repetitive elements which have also been found in *Xanthomonas* and other phytopathogenic bacteria (Louws *et al.*, 1994, 1995). Both approaches are intensively used to assess genetic diversity and as an additional tool for bacterial classification (de Bruijn, 1992). Other molecular methods, including SDS-PAGE and multilocus enzyme electrophoresis are frequently employed in studies of *Xanthomonas* (Vauterin *et al.*, 1991). A more conserved genetic characteristic, the 16S–23S intergenic spacer (IGS) of the rDNA operon, has also been used to infer the phylogenetic relationships and identification of bacteria at different taxonomic levels (see Gürtler & Stanisich, 1996).

In this study we examined the genetic diversity of 55 strains of *X. campestris* pv. *passiflorae* (referred to as *passiflorae* strains) isolated from infected passion fruit leaves collected from different geographic regions of Brazil. The relatedness of a representative strain of 15 clusters generated by RAPD, 16 species of *Xanthomonas* and *Pseudomonas syringae* pv. *passiflorae* was

verified by comparison of the genomic fingerprints generated by RAPD, rep-PCR, RFLP-IGS and SDS-PAGE of whole-cell proteins. A sound taxonomic allocation of the *passiflorae* strains within the genus *Xanthomonas* was obtained by DNA-DNA hybridization.

METHODS

Bacterial strains and culture medium. All *X. campestris* pv. *passiflorae* strains (Table 1) were obtained from the collection of the Instituto Biológico, Section of Phytopathological Bacteriology (IBSBF), Campinas, SP, Brazil, and from the Instituto Agrônômico do Paraná (IAPAR), Londrina, PR, Brazil. The strains were collected from different states in Brazil (Fig. 1). Two strains, 960 and 1171, showed white colonies and were confirmed as pathogens of *Passiflora alata*. For comparison, other type and reference strains from the species of *Xanthomonas* described by Vauterin *et al.* (1995) were also used. The following type and reference strains were purchased from the culture collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium: *Xanthomonas axonopodis* pv. *axonopodis* LMG 538^T, *Xanthomonas bromi* LMG 947^T, *X. campestris* pv. *campestris* LMG 568^T, *Xanthomonas cassavae* LMG 673^T, *Xanthomonas codiae* LMG 8678^T, *Xanthomonas cucurbitae*

LMG 690^T, *Xanthomonas hortorum* pv. *hederiae* LMG 733, *Xanthomonas hyacinthi* LMG 739^T, *Xanthomonas melonis* LMG 8670^T, *Xanthomonas pisi* LMG 847^T, *Xanthomonas oryzae* pv. *oryzae*, LMG 5047, *Xanthomonas sacchari* LMG 471^T, *Xanthomonas theicola* LMG 8684^T, *Xanthomonas translucens* pv. *translucens* LMG 876^T, *Xanthomonas vasicola* pv. *holcicola* LMG 736^T, *Xanthomonas vesicatoria* LMG 911^T and *P. syringae* pv. *passiflorae* LMG 5185. The strains *Xanthomonas albilineans* ICMP 196, *Xanthomonas arboricola* pv. *juglandis* ICMP 35 and *X. campestris* pv. *passiflorae* ICMP 3151 were provided by the International Collection of Microorganisms from Plants (ICMP), Auckland, New Zealand. All strains were grown on NYG medium (Turner *et al.*, 1984) at 30 °C.

DNA extraction. An aliquot (1.5 ml) of bacterial suspension, grown overnight, was washed with 500 µl TAS buffer (50 mM Tris/HCl pH 7.8, 50 mM EDTA, 150 mM NaCl), centrifuged and resuspended in 450 µl of the same buffer. Fifty microlitres of SDS (10 %, w/v) and 100 µg proteinase K were added and the mixture was incubated for 1 h at 55 °C. Proteins and cell debris were extracted with phenol and chloroform and the aqueous suspension was then dialysed against TE buffer (10 mM Tris/HCl pH 7.8, 1 mM EDTA) for 48 h. DNA concentration was estimated by comparison with known concentrations of λ DNA in agarose gel electrophoresis (Sambrook *et al.*, 1989).

Fig. 1. Collection sites of *X. campestris* pv. *passiflorae* strains in Brazil. The Roman numerals refer to the clustering group obtained by RAPD analysis. The state name abbreviations are: BA, Bahia; CE, Ceará; DF, Federal District; MG, Minas Gerais; PR, Paraná; SE, Sergipe; and SP, São Paulo.

PCR amplification. All PCR reactions were performed in a total volume of 25 µl using 20–50 ng DNA. In the RAPD amplification, the primers OPR2, OPR6, OPR8 and OPQ4 (Operon Technologies) were used. The reaction contained 3.75 mM MgCl₂, 100 µM dNTP, 5 µM primer and 0.5 U *Taq* polymerase (BRL). The following cycling conditions were used: 1 × 94 °C for 3 min; 45 × (94 °C for 1 min, 34 °C for 1 min, 72 °C for 2 min). The rep primers (BOX, ERIC, REP) were used as described by Louws *et al.* (1994) with 100 µM dNTP for BOX and 300 µM for ERIC and REP. Amplification of the 16S–23S rDNA IGS was performed using the primers 16S uni330 and 23S uni322anti (Honeycutt *et al.*, 1995). The PCR reaction contained 3.75 mM MgCl₂, 100 µM dNTP, 5 µM primer and 0.5 U *Taq* polymerase (Pharmacia). The cycling conditions were: 1 × 94 °C for 1 min; 40 × (94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min). Control reactions without DNA template were also run. The PCR amplifications were repeated at least twice for most of the strains. The DNA banding patterns obtained following electrophoresis in 1.2% agarose gels were recorded in a binary form (presence/absence). RFLP-IGS patterns were obtained by digestion of the amplification product (3–5 µl) with the restriction enzymes *DdeI*, *HhaI*, *HinfI*, *PstI* and *Sau3AI*. The digested fragments were separated in polyacrylamide gels (10%, w/v) and silver-stained (De Moreno *et al.*, 1985). The 16S rDNA gene, used as a probe to determine DNA concentration in DNA–DNA hybridization experiments, was obtained by PCR amplification of DNA from the strain ICMP 3151. The primers 16F27 and 16R1525 (Hauben *et al.*, 1997) were used in a reaction containing 20–50 ng DNA, 1.5 mM MgCl₂, 100 µM dNTP, 0.5 µM of the primers and 0.5 U *Taq* polymerase (Pharmacia), in a total volume of 25 µl. The sample was cycled under the following conditions: 1 × 94 °C for 2 min; 40 × (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min). The PCR product was purified from low-melting-point agarose gel according to Sambrook *et al.* (1989). In all cases, control reactions lacking DNA template were run.

SDS-PAGE of whole-cell proteins. SDS-PAGE was performed according to Jackman (1985) and Vauterin *et al.* (1991). Freshly grown cells (about 30 mg) were resuspended in TE buffer (10 mM Tris/HCl pH 8, 1 mM EDTA), washed and resuspended in 100 µl of the sample buffer (62.5 mM Tris/HCl pH 8.0, 20% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue). The mixture was heated at 95 °C for 5 min, centrifuged for 10 min and approximately 7 µl of the supernatant was loaded onto the gel. Electrophoresis was performed in a polyacrylamide gel (10 and 4% total acrylamide for the running and stacking gels, respectively). After electrophoresis, the protein bands were visualized by silver-staining (Blum *et al.*, 1987).

Data analysis. Data from the RAPD, rep-PCR and RFLP-IGS were scored in binary form (presence/absence) and similarity matrices were generated using the Jaccard coefficient (*S_j*). Relationships among strains were calculated with the algorithm UPGMA (unweighted pair group method with arithmetic averages) using the software package NTSYS-PC (Applied Biostatistics, Inc.).

DNA–DNA hybridization. Hybridization was performed using nylon membranes (Amersham) and a Hybri slot-blot manifold as described by the manufacturer (Bio-Rad). The probes were labelled using a DIG DNA labelling kit, and the bands were detected with the chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan-4-yl) phenyl phosphate) as

recommended by the manufacturer (Boehringer Mannheim). The signal, detected by exposure to X-ray film (Kodak), was measured using an Ultrascan XL-enhancer laser densitometer (LKB Bromma).

RESULTS

Extensive polymorphism within the *passiflorae* group revealed by RAPD analysis

All *passiflorae* strains were collected from 1968 to 1996 in different states of Brazil (Fig. 1), mainly São Paulo and Paraná, and deposited in the IBSBF and IAPAR collections. The regions shown in Fig. 1 were broadly divided into a Northern region, consisting of the states of Bahia (BA), Ceará (CE), Distrito Federal (DF), Minas Gerais (MG) and Sergipe (SE); and a Southern region consisting of the states of Paraná (PR) and São Paulo (SP).

Preliminary RAPD experiments were conducted with 10 strains, using 13 primers to standardize the PCR conditions and to choose the appropriate primers. Most of the primers yielded no amplification or produced inconsistent PCR products and were not used. Four primers (OPR2, OPR6, OPR8 and OPQ4) were selected to assess the diversity among the 55 *X. campestris* pv. *passiflorae* strains. In the first evaluation, all *X. campestris* pv. *passiflorae* strains and the pathovar reference strain (ICMP 3151) were analysed. The PCR reaction was run at least twice for each strain. Inconsistent bands were checked by performing a third run. The primer OPR2 yielded fragments ranging from 0.3 to 1.6 kb and nine fragments were considered for analysis. A 1.0 kb band was common to all strains and variation was detected with the smaller fragments. Six different patterns were obtained and two major ones were shown by more than 75% of the strains. OPR6 amplified up to four fragments per strain (0.3–2.5 kb) and eight bands were included in the cluster analyses. A 2.5 kb band was shown by all strains. Two patterns were prevalent among the six detected. OPR8 produced greater polymorphism but visualization of the fragments was more difficult. Two bands, of 0.3 and 1.0 kb, were shared by all strains and the bands between these sizes yielded most of the differences among the strains. OPQ4 showed the lowest level of polymorphism, amplifying fragments of 0.5–2.0 kb. Two bands (0.8 and 1.4 kb) were shared by all strains. Five different patterns were detected, one of which was shown by more than 90% of the strains.

In the dendrogram constructed using 34 scored bands from the RAPD data (Fig. 2), few strains showed an identical RAPD pattern and three major clusters included 94% of the strains. One cluster comprised seven strains, isolated from different hosts (*P. edulis*, *P. alata* or *Passiflora* spp.) from the Southern states. A second cluster included 27 strains collected in the same region (Southern) but showed no correlation to the host and only two strains (748 and 621) were from the Northern region. A third cluster included 15 strains

Fig. 2. Clustering (S/UPGMA) obtained from 54 *X. campestris* pv. *passiflorae* strains based on RAPD data for the primers OPR2, OPR6, OPR8 and OPQ4. Scale bar indicates similarity.

from the Northern states; most of these strains were isolated from an unknown host (*Passiflora* sp.). Five strains (714, 1171, 11407, 11331 and 11342) were not grouped and remained separated from the others.

A limit of 70% of similarity was chosen in order to select representative strains of each cluster for further analysis. Accordingly, the 15 strains (I–XV) representing each cluster were: 777, 11324, 120, 121, 1151, 11323, 11407, 11329, 11341, 1171, 11331, 11342, 714, 592 and 717. Fig. 1 shows the distribution of all *passiflorae* strains designated by their cluster number. Clusters I–XII were collected in the Southern states, with a prevalence of strains from cluster IV (20 strains), which was widely distributed in both states (SP and PR). In contrast, strains from clusters XIII–XIV were found exclusively in the Northern region, with a prevalence of strains from cluster XIV (12 strains). These results indicate that the two regions have distinct populations (clusters) with almost no overlapping. Only two strains (748 and 621), from clusters IV and V, respectively, were found in the Northern states.

Relatedness of the selected *passiflorae* strains to different *Xanthomonas* species as assessed by RAPD

In a second evaluation by RAPD, the profiles of the 15 selected *passiflorae* strains and the pathovar reference strain, ICMP 3151, were compared to those obtained with 16 species of *Xanthomonas* and *P. syringae* pv. *passiflorae* using the primers OPR2, OPR6, OPR8 and OPQ4. A high level of polymorphism was detected and each species of *Xanthomonas* yielded a unique genomic

fingerprint. With OPR2, bands ranging from 0.3 to 3.0 kb were obtained and 23 bands were scored. All species showed a distinct profile; in some cases the profiles were similar but differed in at least one major band. OPR6 amplified fragments ranging from 0.5 to 3.0 kb and 20 bands were used in the analysis. This primer did not amplify DNA from *X. pisi* and *X. oryzae*. OPR8 produced fragments between 0.3 and 2.3 kb and 15 bands were scored. With OPQ4, 15 bands varying from 0.4 to 2.8 kb were used in the analysis. A total of 73 bands obtained with the four primers were used to construct the dendrogram based on a UPGMA algorithm (Fig. 3a). Each representative strain of the different *Xanthomonas* species showed a very distinctive pattern and the level of similarity among them was lower than 45%. Some species, such as *X. campestris* and *X. axonopodis*, were closely related to each other (45% similarity) and the *passiflorae* strains were also closer to these two species and to *X. vasicola* and *X. oryzae* (17% similarity) than to the other species (<10% similarity).

rep-PCR fingerprintings of selected *passiflorae* strains and *Xanthomonas* species

The set of rep primers, BOX, ERIC and REP, was used to amplify DNA from 16 *passiflorae* strains, 18 *Xanthomonas* species and *P. syringae* pv. *passiflorae*. Analysis of the individual and combined patterns using the three sets of primers revealed considerable genetic diversity. Amplification with BOX primers yielded 27 scorable bands, ranging from 4.0 to 0.5 kb. Within the *passiflorae* group, some strains showed

Fig. 3. (a) Clustering (S_j /UPGMA) of 16 selected *passiflorae* strains and 16 *Xanthomonas* species based on RAPD data for the primers OPR2, OPR6, OPR8 and OPQ4. (b) Clustering (S_j /UPGMA) obtained for the selected *passiflorae* strains and other *Xanthomonas* species using the combined data from rep-PCR experiments. Scale bar indicates similarity.

identical patterns (3151 = 120 = 121, 1151 = 11329, 1171 = 11407) and even though a reasonable degree of diversity was detected, a unique cluster was formed. Two bands (~ 0.7 and ~ 0.6 kb) were consistently shared by all *passiflorae* strains. ERIC primers yielded a broader range of variation and other *Xanthomonas* species (*cassavae*, *campestris* and *melonis*) were embedded within the *passiflorae* group. A total of 20 bands, most of them varying from 0.5 to 3.0 kb, were considered for cluster analysis. A very intense and high-molecular-mass (5.0 kb) band was observed with *X. arboricola*. Amplification with REP primers showed unique patterns for the *Xanthomonas* species. A total of 16 bands (0.3–3.1 kb) were scored and included in the analysis. Two strains (11329 and 11341) showed

Fig. 4. Dendrogram (S_j /UPGMA) of the RFLP patterns of the 16S–23S rDNA IGS of *passiflorae* strains and other *Xanthomonas* species digested with the restriction enzymes *DdeI*, *HhaI*, *HinfI*, *PstI* and *Sau3AI*. Scale bar indicates similarity.

identical patterns. The *Xanthomonas* species were clearly differentiated from the *passiflorae* strains.

A dendrogram based on the combined data of the rep primers (Fig. 3b) showed that each *passiflorae* strain and each *Xanthomonas* species was identifiable by unique patterns. The genetic diversity within the *passiflorae* group was substantial, with similarities of 38–85%. This group was distantly related to the other *Xanthomonas* species (<15% similarity). The level of relatedness among the *Xanthomonas* species was also very low (<15%).

RFLP-IGS of selected *passiflorae* strains and *Xanthomonas* species

All 16 *passiflorae* strains and most of the *Xanthomonas* species yielded a single PCR product of ~ 1.1 kb. The *Pseudomonas* species showed a higher band (~ 1.2 kb). The IGS products were digested with *DdeI*, *HhaI*, *HinfI*, *PstI* and *Sau3AI* restriction enzymes and the products were separated in polyacrylamide gels (data not shown). A low level of polymorphism was detected within the *passiflorae* group and identical profiles were obtained using *DdeI* or *HinfI*. With *HhaI*, *PstI* and *Sau3AI*, two patterns were obtained with each enzyme: a major pattern shown by most of the strains and a second which was shown by one or two strains. The dendrogram depicted in Fig. 4 was constructed by using 81 different bands, ranging from 20 to ~ 1100 bp. The *passiflorae* strains revealed an unexpected variation of up to 15%. This variation represents differences in one to three bands. Fig. 4 also shows the relationships with the other *Xanthomonas* species. A high degree of polymorphism was detected and the level of similarity ranged from 30 to 87%. The species *X. vasicola* and *X. cucurbitae* and *X. pisi* and *X. bromi*

Fig. 5. SDS-PAGE of whole-cell proteins of (a) *passiflorae* strains and (b) other *Xanthomonas* species plus *P. syringae* pv. *passiflorae*. (a) Lanes: M, 1.0 kb molecular mass markers (Gibco-BRL); 1, ICMP 3151 (reference strain); 2, 777; 3, 120; 4, 121; 5, 714; 6, 1171; 7, 11324; 8, 11323; 9, 11407; 10, 11329; 11, 11341; 12, 11331; 13, 11342; 14, 592; 15, 1151; 16, 717. (b) Lanes: M, 1.0 kb molecular mass markers (Gibco-BRL); 1, ICMP 3151 (*X. campestris* pv. *passiflorae* reference strain); 2, *X. campestris*; 3, *X. axonopodis*; 4, *X. translucens*; 5, *X. hyacinthi*; 6, *X. vesicatoria*; 7, *X. sacchari*; 8, *X. vasicola*; 9, *X. cucurbitae*; 10, *X. melonis*; 11, *X. cassavae*; 12, *X. hortorum*; 13, *X. theicola*; 14, *X. pisi*; 15, *X. bromi*; 16, *X. codiae*; 17, *X. oryzae*; 18, *P. syringae* pv. *passiflorae*.

were closer to each other, whereas *X. theicola*, *X. translucens*, *X. hyacinthi* and *X. sacchari* were more distantly related to other *Xanthomonas* species. The *passiflorae* group showed ~60% similarity to a major cluster which included the following species: *X. axonopodis*, *X. campestris*, *X. vasicola*, *X. cucurbitae*, *X. pisi*, *X. bromi*, *X. cassavae* and *X. hortorum*.

SDS-PAGE of whole-cell proteins

The whole-cell protein patterns of the 16 *passiflorae* strains, 16 species of *Xanthomonas* and *P. syringae* pv. *passiflorae* were analysed by SDS-PAGE (Fig. 5). The protein bands showed a very homogeneous profile within the *passiflorae* strains and most of the bands were shared by all strains. Differences among the strains were most commonly related to the intensity of certain bands (Fig. 5a). In contrast, the protein patterns of the *Xanthomonas* species showed a high level of diversity and there was no identical profile

(Fig. 5b). A band of approximately 60 kDa was common to all *Xanthomonas* strains and a few other bands were considered common to some species. For example, a 52 kDa band was exhibited by the *passiflorae* strains, *X. campestris*, *X. axonopodis*, *X. vasicola*, *X. cassavae*, *X. theicola*, *X. pisi*, *X. bromi* and *X. codiae*. A dendrogram constructed (not shown) showed that the similarity within the *passiflorae* group was very high, ranging from 94 to 100%, and a major cluster included 87.5% of the strains analysed. The *passiflorae* strains were more related to *X. axonopodis*, *X. pisi*, *X. bromi* and *X. hyacinthi*. The different *Xanthomonas* species had a high level of heterogeneity (25–75%). *X. axonopodis*, *X. pisi*, *X. bromi*, *X. hyacinthi*, *X. hortorum*, *X. codiae*, *X. vesicatoria*, *X. vasicola*, *X. campestris*, *X. melonis* and *X. cassavae* showed the highest similarity values, from 50 to 75%. The other *Xanthomonas* species showed lower values (<37%).

DNA–DNA hybridization

The hybridization was performed using DNA from the 16 *passiflorae* strains, 16 species of *Xanthomonas* and *P. syringae* pv. *passiflorae*. Digoxigenin-labelled DNA of the pathovar reference strain (ICMP 3151) was used as a probe and detected by chemiluminescence. Although similar methods have been described previously, most of them have used biotinylated DNA (see Jahnke, 1994). For this reason, the detection limit of the assay and the effect of the amount of DNA were determined. The detection level was tested using different concentrations of DNA (100, 250, 500, 750, 1000 and 2000 ng) from two distantly related species, *X. campestris* pv. *passiflorae* ICMP 3151 and *X. albilineans*. The intensity of the bands was more variable when homologous DNA was used and less than 100 ng of DNA could be detected. However with this amount of DNA no band was seen with non-homologous DNA. For this reason, the amount of DNA used was fixed at 200 ng, which yielded a weak detectable band in non-homologous hybridization. As the amount of DNA had to be measured precisely, different methods were tested. Initially the DNA levels were estimated either by the absorbance at 260 nm or by visual inspection of gels containing a known amount of λ DNA. Since neither of these methods was precise, a finer estimate was obtained by hybridization. A presumed amount of 200 ng DNA was applied onto nylon membranes and hybridization was carried out with digoxigenin-labelled 16S rDNA, obtained by PCR amplification of *X. campestris* pv. *passiflorae* ICMP 3151, as a probe. The homology of the 16S rDNA was assumed to be equivalent in all *Xanthomonas* species because of the high level of similarity of this gene (mean of 98%) within the genus (Hauben *et al.*, 1997). Two nylon membranes (two replicates) were prepared using the estimated 200 ng DNA from all 16 *passiflorae* strains, 17 *Xanthomonas* species and *P. syringae* pv. *passiflorae* and the membranes were then hybridized to the 16S

Fig. 6. DNA-DNA hybridization of *passiflorae* strains (left) and *Xanthomonas* species plus *P. syringae* pv. *passiflorae* (right) with a 16S rDNA probe (a) and with DNA from *passiflorae* ICMP 3151 as a probe (b).

rDNA probe to estimate the DNA concentration of each strain. The bands obtained (Fig. 6a) were read in a densitometer and one band was considered as a control with a defined DNA concentration. Thus, for example, a densitometer reading of 9.7 for the corresponding band of *passiflorae* ICMP 3151 was assumed as containing 200 ng DNA. All other readings were normalized to this control. The two membranes were dehybridized and reused for hybridization with the DNA from *passiflorae* ICMP 3151 used as a probe. The results (Fig. 6b) were read in a densitometer, corrected for the concentration of DNA and are shown in Fig. 7. For hybridizations within the *passiflorae* group, all strains showed homologies of 73–160%. Hybridization of the *passiflorae* ICMP 3151 to other *Xanthomonas* species revealed two groups of DNA homology. One group, with DNA homology values between 39 and 50%, included *X. oryzae*, *X. bromi*, *X. vasicola*, *X. cassavae* and *X. codiae*. The second group (2–27% homology) included all other *Xanthomonas* strains. *P. syringae* pv. *passiflorae* showed 0.65% homology to the *passiflorae* strain. A homology of 67.5% was obtained with *X. axonopodis*. The standard deviation ranged from 0.01 to 6.8%, excluding *X. cassavae*, which presented a considerable variation (18.5%), and *X. melonis* and *X. oryzae* (both ~8.5%). Pairwise comparisons of the binding values between *X. axonopodis* and other species reported by Vauterin *et al.* (1995) with the values obtained here revealed some similarities. The major discrepancies in the homology were related to the species *X. campestris* and *X.*

Fig. 7. DNA-DNA homology (%) of the *passiflorae* ICMP 3151 (used as a probe) compared to different *Xanthomonas* species plus *P. syringae* pv. *passiflorae*.

vesicatoria, which had values lower than 15%. In other cases, the relatedness between *X. axonopodis* and other *Xanthomonas* species was comparable, especially with the more distantly related species. Accordingly, the *Xanthomonas* species *translucens*, *hyacinthi*, *sacchari*, *theicola* and *albilineans* were the least related to *X. axonopodis*. The DNA-DNA hybridizations were repeated to confirm the homology of the *passiflorae* strain to *X. axonopodis* and its relationship to *X. campestris*. The results showed 54% homology of the *passiflorae* strain with *X. axonopodis* and 16% homology with *X. campestris*.

DISCUSSION

Few studies have been conducted with xanthomonads from passion fruit plants and here we present a first survey of strains in this group analysed by RAPD genomic fingerprinting. Extensive genetic diversity was observed and very few of the 55 strains of *X. campestris* pv. *passiflorae* studied were considered to be identical. The high level of polymorphism in these pathogens may be attributed to the long period of existence of *Passiflora* plants in Brazil. *P. edulis* is native in Southern Brazil and was introduced into other countries (Australia, South Africa, New Zealand, Hawaii and Kenya) in the nineteenth century for commercial purposes (see Smith, 1976). Similar diversity has been described for *X. campestris* pv. *manihotis* in South America but is less in the more homogeneous populations of Africa where the *Manihot* plant was introduced more recently (Verdier *et al.*, 1994). Three major clusters, which included most of the strains, and 12 minor clusters were observed in the *passiflorae* strains, based on a minimum level of similarity of ~70%. The pathogenicity of a representative strain of each cluster together with the pathovar reference strain (ICMP 3151) was confirmed by bacterial infiltration on detached leaves of *P. alata*. Two leaves per strain were inoculated with 20–50 µl of

a cell suspension with an OD₆₀₀ of 0.5. All leaves showed a greasy halo 4–7 d after inoculation. No differences were detected in the severity of the symptoms. *X. campestris* LMG 568^T was used as a control and only hypersensitive symptoms were visible after 30 h (data not shown). A correlation between the major clusters and the region of sampling was detected, with a specific cluster being predominant in each region. The genetic differences between the *passiflorae* strains collected in the Northern and Southern regions may indicate either that there is no transport of the plants (or seeds) between the two regions, or that the strains have a high ecological specificity. Indeed, the climate and rainfall conditions in both regions are quite distinct, which could explain the differences in the genomic structure of the populations. However, a more intensive sampling in the Northern region is required to confirm this correlation. Differences in the structure of a bacterial population incited either by geographical barriers (Adhikari *et al.*, 1995; Restrepo & Verdier, 1997) or by the hosts (Saux *et al.*, 1998) have been described. Unfortunately, in the present work the hosts of the strains belonging to clusters XIII–XV were unavailable and the host–pathogen relationship could not be assessed.

Although the rep primers exploit more specific regions in the genome, the existence of a high level of polymorphism was confirmed by the amplification products obtained with these primers. A complex pattern of unique fingerprints was obtained for the 16 selected *passiflorae* strains and the 18 *Xanthomonas* species. The repetitive sequences ERIC and REP are highly conserved in eubacteria (Versalovic *et al.*, 1991; Sharples & Lloyd, 1990), including phytopathogenic bacteria such as *Xanthomonas* and *Pseudomonas* (Louws *et al.*, 1994, 1995). In bacteria interacting with plants, these three sets of repetitive primers have revealed substantial polymorphism at an intra-specific level (Selenska-Pobell *et al.*, 1995; Louws *et al.*, 1994, 1995; Vinuesa *et al.*, 1998). The evolutionary conservation of these repetitive elements in eubacteria has been explained by assuming they have a role in regulating important functions (Higgins *et al.*, 1998). However, the extensive intra-specific level variation has not been explained. Indeed, each strain analysed yielded a strain-specific fingerprint, indicating a high level of changes generating distinct genomic structures. In *Candida albicans* (Scherer & Stevens, 1998), repetitive elements may affect genomic stability by promoting chromosomal rearrangements or by providing hot-spots for recombination. In *Escherichia coli*, 500–100 copies of REP elements are present and because of the high level of homology among them (see Gilson *et al.*, 1987) possibly they can act as homology cassettes. Such variation has also been reported for another repetitive element described by Leach *et al.* (1992).

The RFLP-IGS analysis, although performed with a limited number of restriction enzymes, was still able to detect diversity within the *passiflorae* group. In general,

analysis of the 16S rDNA rather than the IGS has been employed more frequently in microbial systematics and phylogeny studies (Gutell *et al.*, 1994). However Hauben *et al.* (1997) have shown that the extreme similarity in the 16S rDNA sequences of *Xanthomonas* species imposes limitations on attempts to establish a comprehensive view of the relationships among the different species.

In the present work, all 16 species of *Xanthomonas* were differentiated by the RFLP-IGS pattern, although the relationships among them did not reflect the relationships obtained by DNA–DNA hybridization (Vauterin *et al.*, 1995) or 16S rDNA analysis (Hauben *et al.*, 1997). However, the data were comparable when distant species were considered. Accordingly, the *X. albilineans* (comprising *X. translucens*, *X. hyacinthi* and *X. theicola*) and the *X. sacchari* cores are more distantly related to the other *Xanthomonas* species (the *X. campestris* core). Although only a few strains representing different *Xanthomonas* species were tested in the present work, the level of differentiation among them was striking. Indeed, the RFLP-IGS pattern using only three restriction enzymes, *Dde*I, *Hinf*I and *Sau*3AI, was efficient in discriminating all 16 species of *Xanthomonas*. The *passiflorae* group could not be allocated to any *Xanthomonas* species based on the RFLP pattern and may therefore represent a new species. The species most related to the *passiflorae* strains were *X. campestris* and *X. axonopodis*, although the level of similarity was low (~59%). Indeed, the similarity between *X. vasicola* and *X. cucurbitae*, for example, was higher (89%) than the similarity of the *passiflorae* group to any other *Xanthomonas* species (<60%). Since it is not yet possible to establish the limits of RFLP-IGS in unambiguously delineating taxa at the species level, a greater number of strains representing each *Xanthomonas* species needs to be examined and the 16S–23S rDNA IGS sequences determined in order to establish the usefulness of this genomic region as a marker of genetic diversity and/or as a taxonomic tool. The heterogeneity in the 16S–23S rDNA IGS has been attributed to the number, length and composition of these spacers and it has been exploited to differentiate various bacterial species or strains (see Gürtler & Stanisich, 1996). Although the 16S–23S rDNA IGS of *Xanthomonas* has not been analysed in detail, it is likely that a higher level of heterogeneity exists in this region than in the conserved 16S rDNA. A more detailed analysis of this region in *Xanthomonas* species may reveal the extent of the differences observed and also would allow the construction of a phylogenetic tree more consistent with the DNA–DNA hybridization data by defining more clearly the phylogenetic relationships of closely related species.

SDS-PAGE of whole-cell proteins revealed a very uniform pattern within the *passiflorae* group, as described for other pathovars of *Xanthomonas* (see Vauterin *et al.*, 1991). In contrast, discriminating patterns were shown by different species of *Xantho-*

monas. Since the *passiflorae* group was closely related to a major cluster comprising various *Xanthomonas* species, the taxonomic position of this group could not be assigned with certainty.

A general comparison of the approaches used in this study shows that they are not comparable since clustering obtained by one method was disrupted when another method was used. The results were therefore not sufficiently consistent to allow a taxonomic allocation of the *passiflorae* strains within the genus *Xanthomonas*. DNA–DNA hybridization, however, allocated this group to *X. axonopodis* with a mean homology of 60.7%. This species is the largest and most genomically heterogeneous group in the *Xanthomonas* genus and includes 34 former *X. campestris* pathovars and *X. axonopodis* (Vauterin *et al.*, 1995). Based on these results, a new designation of *X. campestris* pv. *passiflorae* as *X. axonopodis* pv. *passiflorae* is suggested. The pathovar characterization described by Pereira (1969) is maintained.

ACKNOWLEDGEMENTS

This work was supported by FAPESP (Proc. 90/7067-1). CNPq sponsored a studentship to E.R.G. The authors thank Dr L. O. S. Beriam and Dr J. Rodrigues Neto (IB/SBF) and Dr R. P. Leite (IAPAR) for providing the *passiflorae* strains, and Dr S. Hysiop for revising the English.

REFERENCES

- Adhikari, T. B., Vera Cruz, C. M., Zhang, Q., Nelson, R. J., Skinner, D. Z., Mew, T. W. & Leach, J. E. (1995). Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. *Appl Environ Microbiol* **61**, 966–971.
- Blum, H., Beier, H. & Gross, J. J. (1987). Improved silver staining of plant protein, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–98.
- de Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl Environ Microbiol* **58**, 2180–2187.
- De Moreno, M. R., Smith, J. F. & Smith, R. V. (1985). Silver staining of proteins in polyacrylamide gels: increased sensitivity through a combined Coomassie blue–silver stain procedure. *Anal Biochem* **151**, 466–470.
- Gilson, E., Rousset, J. P., Clement, J. M. & Hofnung, M. (1987). Palindromic units: a case of highly repetitive DNA sequences in bacteria. *Trends Genet* **3**, 226–230.
- Gürtler, V. & Stanisch, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
- Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S structures from a comparative perspective. *Microbiol Rev* **58**, 10–26.
- Hauben, L., Vauterin, L., Swings, J. & Moore, E. R. B. (1997). Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int J Syst Bacteriol* **47**, 328–335.
- Higgins, C. F., McLaren, R. S. & Newbury, S. F. (1998). Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? – a review. *Gene* **72**, 3–14.
- Honeycutt, R. J., Sobral, B. W. S. & McClelland, M. (1995). tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. *Microbiology* **141**, 3229–3239.
- Jackman, P. J. H. (1985). Bacterial taxonomy based on electrophoretic whole-cell protein patterns. In *Chemical Methods in Bacterial Systematics*, pp. 115–129. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Jahnke, D. D. (1994). A modified method of quantitative colorimetric DNA–DNA hybridization on membrane filters for bacterial identification. *J Microbiol Methods* **20**, 273–288.
- Leach, J. E., Rhoads, M. L., Vera Cruz, C. M., White, F. F., Mew, T. W. & Leung, H. (1992). Assessment of genetic diversity and population structure of *Xanthomonas oryzae* pv. *oryzae* with a repetitive DNA element. *Appl Environ Microbiol* **58**, 2188–2195.
- Louws, F. J., Fulbright, D. W., Stephens, C. T. & de Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol* **60**, 2286–2295.
- Louws, F. J., Fulbright, D. W., Stephens, C. T. & de Bruijn, F. J. (1995). Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* **85**, 528–536.
- Pereira, A. L. G. (1969). Uma nova doença bacteriana do maracujá (*Passiflora edulis*, Sims) causada por *Xanthomonas passiflorae* n.sp. *Arq Inst Biol* **36**, 163–174.
- Restrepo, S. & Verdier, V. (1997). Geographical differentiation of the population of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. *Appl Environ Microbiol* **63**, 4427–4434.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Saux, M. F.-L., Mauléon, H., Constant, P., Brunel, B. & Boemare, N. (1998). PCR-ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode hosts. *Appl Environ Microbiol* **64**, 4246–4254.
- Scherer, S. & Stevens, D. A. (1998). A *Candida albicans* dispersed, repeated gene family and its epidemiologic applications. *Proc Natl Acad Sci USA* **85**, 1452–1456.
- Selenska-Pobell, S., Gigova, L. & Petrova, N. (1995). Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. *J Appl Bacteriol* **79**, 425–431.
- Sharples, G. P. & Lloyd, R. G. (1990). A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Res* **18**, 6503–6508.
- Smith, P. M. (1976). Minor crops. In *Evolution of Crop Plants*, pp. 301–304. Edited by N. W. Simmonds. New York: Longman.
- Souza, J. S. I. & Meletti, L. M. M. (1997). *Maracujá: Espécies, Variedades, Cultivo*, 1st edn. FEALQ, Piracicaba, Brazil: Fundação de Estudos Agrários Luiz de Queiroz.
- Teixeira, C. G. (1994). Cultura. In *Maracujá: Cultura, Matéria Prima, Processamento e Aspectos Econômicos*, pp. 1–142 (Série Frutas Tropicais, 9). Campinas, Brazil: Instituto de Tecnologia de Alimentos.
- Torres Filho, J. & Ponte, J. J. (1994). Estudo sobre o controle da bacteriose ou “morte precoce” (*Xanthomonas campestris* pv.

- passiflorae*) do maracujá amarelo (*Passiflora edulis* f. *flavicarpa*). *Fitopatol Bras* **18**, 34–38.
- Turner, T., Barber, C. & Daniels, M. (1984). Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. *Mol Gen Genet* **195**, 101–107.
- Vauterin, L., Swings, J. & Kersters, K. (1991). Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. *J Gen Microbiol* **137**, 1677–1687.
- Vauterin, L., Hoste, B., Kersters, K. & Swings, J. (1995). Reclassification of *Xanthomonas*. *Int J Syst Bacteriol* **45**, 472–489.
- Verdier, V., Boher, B., Maraite, H. & Geiger, J. P. (1994). Pathological and molecular characterization of *Xanthomonas campestris* strains causing diseases of cassava (*Manihot esculenta*). *Appl Environ Microbiol* **60**, 4478–4486.
- Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.
- Vinuesa, P., Rademaker, J. L. W., de Bruijn, F. J. & Werner, D. (1998). Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S–23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Appl Environ Microbiol* **64**, 2096–2104.
- Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.
- Young, J. M., Saddler, G. S., Takikawa, Y., De Boer, S. H., Vauterin, L., Gardan, L., Gvozdyak, R. I. & Stead, D. E. (1996). Names of plant pathogenic bacteria 1864–1995. *Rev Plant Pathol* **75**, 721–763.