Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins

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A numerical analysis was performed on SDS-PAGE protein patterns of 307 *Xanthomonas* strains comprising all species and 27 *X. campestris* pathovars. The electrophoretic groupings corresponded in some, but not all cases with the existing pathovars. Six pathovars constituted distinct entities, comprising all strains investigated. These included *X. campestris* pv. *campestris*, *X. campestris* pv. *graminis*, *X. campestris* pv. *hyacinthi*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *pruni* and *X. campestris* pv. *theicola*. A great number of pathovars consisted of a homogeneous group from which only one or a few strains were aberrant. In two cases (*X. campestris* pv. *ricini* and *X. campestris* pv. *vitians*) even the pathovar reference strain was aberrant. Six pathovars from members of the plant family *Fabaceae* could not be differentiated from one another: *X. campestris* pv. *phaseoli*, *X. campestris* pv. *phaseoli* var. *fuscans*, *X. campestris* pv. *cajani*, *X. campestris* pv. *vignicola*, *X. campestris* pv. *alfalfae* and *X. campestris* pv. *glycines*. At least six *X. campestris* pathovars were heterogeneous, displaying two or more protein electrophoretic types: *X. campestris* pv. *alfalfae*, *X. campestris* pv. *dieffenbachiae*, *X. campestris* pv. *juglandis*, *X. campestris* pv. *poinsettiicola*, *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *vignicola*. A database of SDS-protein patterns provides a valuable tool for the identification of unknown xanthomonads.

Introduction

The bacterial genus *Xanthomonas* consists of numerous plant pathogens, occurring worldwide and causing disease on diverse economically important crops (Bradbury, 1984; Kennedy & Alcorn, 1980; Leyns *et al.*, 1984). Whereas the determination of the genus *Xanthomonas* and its species poses no great problem, the characterization of the *X. campestris* pathovars is still difficult (Ercolani, 1987; Vauterin *et al.*, 1990b). In phytopathological practice, however, there is a need for rapid and unambiguous identification of pathovars of *X. campestris*. These pathovars, which are defined by host or symptom specificity, can usually not be differentiated by other phenotypic features (Dye, 1962; Van den Mooter & Swings, 1990). A number of other techniques have been applied to differentiate pathovars of *X. campestris*, such as typing with polyclonal and monoclonal antibodies, gas chromatographic analysis of cellular fatty acids, protein electrophoresis and DNA probe techniques. Development of rapid high-resolution fingerprinting techniques such as protein electrophoresis in combination with computer-assisted processing of profiles makes it possible to characterize and compare large numbers of strains at the infrasubspecific level (Kerstens, 1985;

Jackman, 1987). Protein electrophoresis was first used to differentiate *Xanthomonas* strains by El-Sharkawy & Huisinigh (1971). Later, protein electrophoretic studies of *Xanthomonas* allowed differentiation of *X. campestris* pv. *oryzae* and pv. *oryzicola* (Vera Cruz *et al.*, 1984), pv. *manihotis* and pv. *cassavae* (Van den Mooter *et al.*, 1987a), and pv. *graminis*, pv. *phleipratensis*, pv. *poae* and pv. *arrhenatheri* (Van den Mooter *et al.*, 1987b). Van Zyl & Steyn (1990) used SDS-PAGE to differentiate *Pseudomonas* and *Xanthomonas* species. A good agreement between groupings obtained by SDS-PAGE, gas chromatographic analysis of cellular fatty acids and DNA–DNA hybridization was found for a number of *X. campestris* pathovars (Kerstens *et al.*, 1989; Stead, 1989; Vauterin *et al.*, 1990b).

In our laboratory, a database of patterns of proteins separated by SDS-PAGE of *Xanthomonas* has been generated, now comprising over 900 strains. In the present paper we report on a numerical comparison of electrophoretic protein patterns of 307 strains comprising 27 *X. campestris* pathovars and representative strains of the other *Xanthomonas* species. Attention was paid to the inclusion of isolates representing the broad geographical distribution and phytopathological diversity of each pathovar. The main objectives of this work were (1)

to examine whether the groupings obtained by SDS-PAGE support the existing pathovar division of *X. campestris* as established by Dye *et al.* (1980); (2) to check the homogeneity or heterogeneity of each of the pathovars studied, and (3) to constitute a database of SDS-PAGE protein patterns on computer files for rapid identification.

Methods

Bacterial cells. All the strains used are listed in Table 1. For each of the species and pathovars studied, the type strain or pathovar reference strain was included. Strains were grown on GYCA medium (Dye, 1962), modified as follows: 1% (w/v) glucose, 0.5% (w/v) yeast extract, 3% (w/v) CaCO₃, and 2% (w/v) agar in distilled water. Stock cultures were maintained at 2 °C.

SDS-PAGE of whole-cell proteins. Cultures were grown on GYCA slants for 48 h at 28 °C. Cells were suspended in 0.01 M-potassium phosphate buffer pH 7.0 and transferred to Roux flasks containing 150 ml GYCA medium. Flasks were incubated for 48 h at 28 °C.

Preparation and electrophoresis of whole-cell protein extracts was performed by the SDS-PAGE method of Laemmli (1970), using a slightly modified procedure. Cells were harvested from the flasks with 20 ml phosphate-buffered saline (4.26 g Na₂HPO₄ l⁻¹, 2.27 g KH₂PO₄ l⁻¹, 8 g NaCl l⁻¹, in distilled water), and washed and centrifuged at 15000 r.p.m. twice. About 70–80 mg of the pellet was suspended in sample treatment buffer (7.5 g Tris l⁻¹, 50 ml β-mercaptoethanol l⁻¹, 100 ml glycerol l⁻¹, in double-distilled water; final pH 6.8, adjusted with HCl) in an Eppendorf tube; 0.14 ml of a 20% (w/v) SDS solution was added, and the suspension was mixed and heated at 95 °C for 10 min. Tubes were cooled on ice and centrifuged at 10000 r.p.m. Supernatant extracts were divided into two portions, one of which was stored at –20 °C for immediate use and the other at –80 °C for long storage.

Electrophoresis was performed in a 12% (w/v) gel slab, run vertically (6 mA constant current, 19 °C) until the bromophenol blue tracking dye had migrated 95 mm (about 15 h). Gels were stained for 1 h in a solution containing 0.25% (w/v) Serva Blue R, 50% (v/v) ethanol and 10% (v/v) acetic acid, and destained in a solution containing 25% methanol and 10% acetic acid. Gels were dried in a Bio-Rad 224 slab gel dryer.

The reproducibility of the electrophoresis technique was monitored by preparing protein extracts of over 40 strains in duplicate and running reference samples on each of the slab gels.

Densitometric recording and normalization of electrophoregrams. The dried stained gels were scanned with an LKB laser densitometer linked to a microcomputer. Densitometric records of 1000 points were normalized and reduced to 400 points as described by Pot *et al.* (1989).

Secondary alignment of traces and numerical analysis. Numerical analysis was performed on a Siemens BS2000 mainframe computer. A Fortran 77 program was developed to perform numerical analysis of up to 800 traces (L. Vauterin, unpublished data). The program calculated the similarity between traces by the Pearson product-moment correlation coefficient (*r*) (Sneath & Sokal, 1973). To optimize matching between pairs of traces, the program performed a secondary alignment of traces during numerical analysis. This alignment involved a lateral displacement of one trace up to five points on either side of the initial alignment. The highest *r*-value was retained. For a given pair of traces, the procedure involved the following steps: (1) calculation of the

initial correlation at the original alignment; (2) displacement of one trace with respect to the other by a single point step forward and recalculation of the correlation; (3) if the new correlation was higher than the initial one, further single-point steps forward were performed until the maximum of correlation was reached. If the correlation value obtained in (2) was lower than the initial one, the routine was executed again from step (2) backward from the original alignment.

Cluster analysis was performed on the matrix of correlation values by the unweighted pair group method using arithmetic averages (UPGMA).

Results and Discussion

The secondary alignment of the standardized traces during numerical analysis improved the reliability of the cluster analysis considerably, even though the protein patterns were highly standardized. An increase of correlation of 17% was observed between patterns of *X. campestris* pv. *hyacinthi* strains. A similar optimization has been applied by other investigators (Albritton *et al.*, 1988; Costas *et al.*, 1989; Jackman *et al.*, 1983). The overall reproducibility of the electrophoresis technique averaged *r* = 0.94.

The dendrogram obtained after numerical analysis of protein electrophoregrams of 307 *Xanthomonas* strains is shown in Fig. 1. In this simplified version, homogeneous groups are represented by triangles. Nineteen clusters of at least three strains clustering at *r* = 0.85 were delineated. Strains and corresponding clusters are listed in Table 1. In the discussion hereafter the location of the pathovar reference strain is only mentioned when it grouped apart from its electrophoretic cluster, or when the pathovar consisted of different clusters.

From the fairly high similarity values between clusters of *Xanthomonas* species and *X. campestris* pathovars it appears that most of the xanthomonads display a common SDS-PAGE protein profile (see also the electrophoregrams in Fig. 2). Highly distinct profiles were only produced by *X. albilineans* and *X. maltophilia*. The species *X. axonopodis*, *X. fragariae* and *X. oryzae* (both pv. *oryzae* and pv. *oryzicola*) could be easily distinguished from each other and from the *X. campestris* pathovars, whereas *X. populi* was rather similar to *X. campestris*.

From 286 *X. campestris* strains studied, 253 (i.e. 88%) grouped together above *r* = 0.70 (clusters 1 to 12). Clearly distinct from this group were *X. campestris* pv. *melonis* (cluster 19), pv. *graminis* Egli & Schmidt 1982 (cluster 17) and pv. *theicola* (cluster 16).

X. campestris pv. *graminis* Egli & Schmidt 1982 is a remarkably distinct and homogeneous pathovar. In previous studies this pathovar could be distinguished from other *X. campestris* pathovars including xanthomonads from grasses and cereals (Van den Mooter & Swings, 1990; Van den Mooter *et al.*, 1987a). All strains

Table 1. Source and cluster assignment of the strains studied

ATCC, American Type Culture Collection, Rockville, Maryland, USA; CNBP, Collection Nationale de Bactéries Phytopathogènes, Angers, France; ICMP, International Collection of Microorganisms from Plants, Auckland, New Zealand; ICPB, International Collection of Phytopathogenic Bacteria, Davis, California; IPO, Instituut voor Plantenziektenkundig onderzoek, Wageningen, The Netherlands; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; PD, Plantenziektenkundige Dienst, Wageningen, The Netherlands.

Species/pathovar	Strains
<i>X. albilineans</i>	
Unclustered	LMG 487 (NCPPB 1050), LMG 490 (NCPPB 2247), LMG 494 ^T (NCPPB 2969)
<i>X. axonopodis</i>	
Unclustered	LMG 538 ^T (NCPPB 457), LMG 540 (NCPPB 2376), LMG 537 (ICMP 74)
<i>X. campestris</i> pv. <i>alfalfae</i>	
Cluster 3a	LMG 8019 (NCPPB 1821), LMG 8020 (NCPPB 2393)
Cluster 3c	LMG 8078 (ICMP 330), LMG 8079 (ICMP 3376), LMG 8080 (ICMP 4765)
Unclustered	LMG 495 (ICPB XA121), LMG 497* (NCPPB 2062), LMG 8018 (NCPPB 480)
<i>X. campestris</i> pv. <i>begoniae</i>	
Cluster 11	LMG 552 (NCPPB 2605), LMG 7190 (NCPPB 1925), LMG 7194 (CNBP 1422), LMG 7196 (CNBP 1424), LMG 7594 (Vantomme Xb1809), LMG 7596 (Vantomme Xb3007), LMG 7597 (Vantomme Xb2208), LMG 7595 (Vantomme Xb1207), LMG 7226 (ATCC 8718), LMG 7193 (CNBP 1421), LMG 7227 (ATCC 11726)
Unclustered	LMG 7303* (ICMP 194), LMG 7307 (ICMP 6652), LMG 7188 (NCPPB 109), LMG 7304 (ICMP 195)
<i>X. campestris</i> pv. <i>cajani</i>	
Cluster 3a	LMG 558* (NCPPB 573), LMG 7387 (NCPPB 2063), LMG 7473 (ICMP 1625)
<i>X. campestris</i> pv. <i>campestris</i>	
Cluster 1	LMG 8121 (ICMP 6537), LMG 8100 (ICMP 4013), LMG 8123 (ICMP 6542), LMG 8099 (ICMP 3984), LMG 583 (Maraite HMB142), LMG 8051 (PD 923), LMG 8003 (NCPPB 1043), LMG 8035 (CFBP 1869), LMG 8102 (ICMP 4578), LMG 8001 (NCPPB 529), LMG 7516 (ICMP 5566), LMG 8112 (ICMP 5571), LMG 571 (NCPPB 1144), LMG 568 ^T (NCPPB 528), LMG 7662 (Vantomme Xc0611)
<i>X. campestris</i> pv. <i>cassavae</i>	
Cluster 5	LMG 8048 (PD 771), LMG 8049 (PD 776), LMG 670 (ICPB XC110), LMG 673* (Maraite HMB54), LMG 671 (Maraite HMB29), LMG 672 (Maraite HMB38), LMG 764 (Maraite HMB59), LMG 5268 (Maraite HMB45), LMG 5269 (Maraite HMB51), LMG 5270 (Maraite HMB53), LMG 5246 (Maraite HMB179), LMG 5265 (Maraite HMB41), LMG 5264 (Maraite HMB37), LMG 5267 (Maraite HMB44)
Unclustered	LMG 5271 (Maraite HMB146)
<i>X. campestris</i> pv. <i>citri</i>	
Cluster 6	LMG 682* (NCPPB 409), LMG 680 (NCPPB 405), LMG 683 (NCPPB 410), LMG 681 (NCPPB 408), LMG 8650 (ICMP 28), LMG 8652 (ICMP 594), LMG 8653 (ICMP 5809), LMG 8654 (ICMP 7494), LMG 8657 (ICMP 8436), LMG 8651 (ICMP 31), LMG 9176 (Civerolo XC59), LMG 9177 (Civerolo XC62), LMG 9184 (Civerolo XC115)
Unclustered	LMG 8655 (ICMP 8432), LMG 8656 (ICMP 8435)
<i>X. campestris</i> pv. <i>cucurbitae</i>	
Cluster 3b	LMG 7479 (ICMP 203)
Cluster 10	LMG 7480 (ICMP 1858), LMG 7481 (ICMP 4766), LMG 690* (NCPPB 2597), LMG 8662 (ICMP 2309), LMG 8661 (ICMP 202), LMG 8663 (ICMP 4769)
<i>X. campestris</i> pv. <i>dieffenbachiae</i>	
Cluster 3e	LMG 7399 (NCPPB 985), LMG 7400 (NCPPB 986)
Cluster 8	LMG 695* (NCPPB 1833), LMG 7484 (ICMP 4656), LMG 8664 (ICMP 7461)

Table 1-*contd.*

Species/pathovar	Strains
<i>X. campestris</i> pv. <i>glycines</i>	
Cluster 3a	LMG 712* (NCPPB 554), LMG 7403 (NCPPB 1124), LMG 8023 (NCPPB 1135), LMG 7404 (NCPPB 1717), LMG 8026 (NCPPB 1714), LMG 8025 (NCPPB 1141), LMG 8125 (ICMP 246), LMG 8126 (ICMP 3415), LMG 8127 (ICMP 3514), LMG 7488 (ICMP 3434)
Cluster 7	LMG 8027 (NCPPB 1716)
Unclustered	LMG 8128 (ICMP 3536)
<i>X. campestris</i> pv. <i>graminis</i> Egli & Schmidt 1982	
Cluster 17	LMG 726* (NCPPB 2700), LMG 607 (Leyns Xg81-28), LMG 713 (Van den Mooter 218), LMG 600 (Leyns Xg81-16), LMG 587 (IPO 118), LMG 595 (Egli E741), LMG 7406 (NCPPB 2696), LMG 7408 (NCPPB 2701), LMG 593 (Egli E733), LMG 7407 (NCPPB 2698)
<i>X. campestris</i> pv. <i>hederae</i>	
Cluster 7e	LMG 733* (NCPPB 939), LMG 734 (ICPB XH1), LMG 7414 (NCPPB 2336), LMG 7413 (NCPPB 2016), LMG 8666 (ICMP 1663), LMG 8665 (ICMP 1406)
Unclustered	LMG 7411 (NCPPB 987), LMG 8668 (ICMP 9510), LMG 8667 (ICMP 9508)
<i>X. campestris</i> pv. <i>hyacinthi</i>	
Cluster 4	LMG 7419 (NCPPB 205), LMG 739* (NCPPB 599), LMG 740 (NCPPB 1622), LMG 7420 (NCPPB 2183), LMG 8041 (PD 205), LMG 8044 (PD 281), LMG 8042 (PD 357), LMG 8043 (PD 280), LMG 742 (ICPB XH110), LMG 741 (NCPPB 2095)
<i>X. campestris</i> pv. <i>juglandis</i>	
Cluster 2	LMG 8045 (PD 130), LMG 8046 (PD 157), LMG 8047 (PD 189)
Cluster 7a	LMG 751 (NCPPB 1447), LMG 748 (NCPPB 412), LMG 750 (NCPPB 414), LMG 752 (NCPPB 1659), LMG 746 (NCPPB 362), LMG 747* (NCPPB 411)
<i>X. campestris</i> pv. <i>malvacearum</i>	
Cluster 3e	LMG 7430 (NCPPB 1699), LMG 7428 (NCPPB 1697)
Cluster 3f	LMG 759 (Marait HMB24), LMG 761* (NCPPB 633), LMG 760 (NCPPB 210), LMG 7429 (NCPPB 1698), LMG 7427 (NCPPB 1048), LMG 762 (NCPPB 2837), LMG 764 (NCPPB 2840), LMG 763 (NCPPB 2838)
Cluster 7c	LMG 7426 (NCPPB 293)
<i>X. campestris</i> pv. <i>manihotis</i>	
Cluster 9	LMG 784 (Marait HMB55), LMG 766 (Marait HMB3), LMG 769 (Marait HMB10), LMG 779 (Marait HMB80), LMG 767 (Marait HMB6), LMG 782 (Marait HMB93), LMG 768 (Marait HMB9), LMG 783 (Marait HMB148), LMG 780 (Marait HMB81), LMG 771 (Marait HMB25), LMG 777 (Marait HMB78), LMG 773* (NCPPB 1834), LMG 774 (Marait HMB60)
Unclustered	LMG 778 (Marait HMB79)
<i>X. campestris</i> pv. <i>melonis</i>	
Cluster 19	LMG 8673 (ICMP 8688), LMG 8670* (ICMP 8682), LMG 8672 (ICMP 8685)
Unclustered	LMG 8671 (ICMP 8683), LMG 8674 (ICMP 9284)
<i>X. campestris</i> pv. <i>pelargonii</i>	
Cluster 12	LMG 7708 (Vantomme Xp1025), LMG 7691 (Vantomme Xp1000), LMG 7317 (ICMP 5919), LMG 7715 (Vantomme Xp1031), LMG 7318 (ICMP 6758), LMG 7690 (Vantomme Xp0904), LMG 7721 (Vantomme Xp1039), LMG 7763 (Vantomme Xp1040), LMG 7764 (Vantomme Xp1041), LMG 7312 (ICMP 230), LMG 7353 (ICMP 229), LMG 7315 (ICMP 5909), LMG 7316 (ICMP 5915), LMG 7354 (ICMP 241), LMG 7314* (ICMP 4321)
<i>X. campestris</i> pv. <i>phaseoli</i>	
Cluster 3a	LMG 842 (Saettler XpNcb), LMG 8011 (NCPPB 1646), LMG 8015 (NCPPB 2064), LMG 7455* (NCPPB 3035), LMG 822 (Saettler Xp12), LMG 823 (Saettler Xp21), LMG 827 (IPO 382), LMG 836 (NCPPB 1420), LMG 8014 (NCPPB 1811)
Cluster 3b	LMG 830 (NCPPB 557), LMG 821 (Saettler Xp11), LMG 829 (NCPPB 556)

Table 1-*contd.*

Species/pathovar	Strains
<i>X. campestris</i> pv. <i>phaseoli</i> (var. <i>fuscans</i>)	
Cluster 3a	LMG 8036 (CFBP 1505), LMG 8037 (CFBP 1815), LMG 8017 (NCPPB 1495), LMG 7456 (NCPPB 456), LMG 7457 (NCPPB 1056), LMG 7458 (NCPPB 1158), LMG 8016 (NCPPB 1433), LMG 824 (PD 24), LMG 8038 (PD 24), LMG 8040 (PD 730), LMG 8039 (PD 365), LMG 841 (NCPPB 2665), LMG 832 (NCPPB 670), LMG 7512 (ICMP 5807), LMG 838 (NCPPB 2033), LMG 826 (NCPPB 381), LMG 7459 (NCPPB 1402), LMG 837 (NCPPB 1654)
Unclustered	LMG 7511 (ICMP 242), LMG 825 (Maraite HMB144), LMG 666 (Saettler Xpfwv)
<i>X. campestris</i> pv. <i>pisi</i>	
Unclustered	LMG 847* (NCPPB 762)
<i>X. campestris</i> pv. <i>poinsetticola</i>	
Cluster 3c	LMG 8677 (ICMP 9512), LMG 8678 (ICMP 9513)
Cluster 7c	LMG 5402 (ICMP 3279)
Cluster 19	LMG 7436 (NCPPB 1939), LMG 8675 (ICMP 3280)
Unclustered	LMG 5403 (ICMP 6274), LMG 8676 (ICMP 7180), LMG 849* (ICPB XP137), LMG 5401 (ICMP 332)
<i>X. campestris</i> pv. <i>pruni</i>	
Cluster 7c	LMG 850 (ICPB XP10), LMG 852* (NCPPB 416), LMG 851 (NCPPB 273), LMG 853 (NCPPB 418), LMG 8681 (ICMP 6677), LMG 854 (NCPPB 419), LMG 856 (NCPPB 923), LMG 855 (NCPPB 420), LMG 857 (NCPPB 924), LMG 858 (NCPPB 926), LMG 8679 (ICMP 86), LMG 7437 (NCPPB 272), LMG 7438 (NCPPB 368), LMG 8680 (ICMP 4100)
<i>X. campestris</i> pv. <i>ricini</i>	
Cluster 3c	LMG 862 (NCPPB 1324), LMG 864 (NCPPB 2065), LMG 7442 (NCPPB 535), LMG 7443 (NCPPB 1473), LMG 7444 (NCPPB 1684), LMG 7440 (NCPPB 113), LMG 8683 (ICMP 7463)
Unclustered	LMG 861* (NCPPB 1063), LMG 7441 (NCPPB 488), LMG 8682 (ICMP 250)
<i>X. campestris</i> pv. <i>theicola</i>	
Cluster 16	LMG 8684* (ICMP 6774), LMG 8685 (ICMP 7291), LMG 8686 (ICMP 7294)
<i>X. campestris</i> pv. <i>vesicatoria</i>	
Cluster 3d	LMG 667 (ICMP 4799), LMG 668 (ICMP 6061), LMG 909 (Maraite HMB108), LMG 910 (Maraite HMB128), LMG 913 (Maraite HMB140), LMG 905 (Kishun 82-2), LMG 914 (Maraite HMB141), LMG 906 (Kishun 82-3), LMG 922 (NCPPB 941), LMG 929 (NCPPB 2573)
Cluster 7	LMG 920 (NCPPB 701)
Cluster 7b	LMG 917 (NCPPB 424), LMG 916 (NCPPB 423), LMG 935 (ICMP 118), LMG 925 (NCPPB 1431), LMG 911* (Maraite HMB 130), LMG 919 (NCPPB 509)
<i>X. campestris</i> pv. <i>vignicola</i>	
Cluster 3a	LMG 828 (NCPPB 555), LMG 831 (NCPPB 638), LMG 839 (NCPPB 2059), LMG 840 (NCPPB 2061), LMG 8138 (ICMP 590)
Cluster 3e	LMG 8136 (ICMP 334), LMG 8137 (ICMP 335), LMG 8139 (ICMP 3389), LMG 8140 (ICMP 3479), LMG 8752* (NCPPB 1838)
<i>X. campestris</i> pv. <i>vitians</i>	
Cluster 6	LMG 8689 (ICMP 6656), LMG 937* (NCPPB 976);
Cluster 7d	LMG 538 (NCPPB 2248), LMG 7452 (NCPPB 970), LMG 7508 (ICMP 4165), LMG 8687 (ICMP 355), LMG 7509 (ICMP 5854), LMG 8688 (ICMP 6461), LMG 8690 (ICMP 6857), LMG 7510 (ICMP 6655)
<i>X. fragariae</i>	
Cluster 18	LMG 707 (Benaki F8), LMG 708 [†] (NCPPB 1469), LMG 709 (NCPPB 1822)
<i>X. maltophilia</i>	
Unclustered	LMG 978 (NCPPB 323), LMG 958 [†] (ATCC 13637), LMG 961 (ATCC 13636)
<i>X. oryzae</i> pv. <i>oryzae</i>	
Cluster 14	LMG 5047 [†] (ICMP 3125), LMG 9602 (PXO 35), LMG 641 (ICMP 4646)
<i>X. oryzae</i> pv. <i>oryzicola</i>	
Cluster 15	LMG 797* (NCPPB 1585), LMG 9646 (BLS 127), LMG 661 (ICMP 3134)
<i>X. populi</i>	
Cluster 13	LMG 5743 [†] (CNBP 1817), LMG 5751 (CNBP 1830), LMG 5750 (CNBP 1829)

* Pathovar reference strain.

[†] Type strain.

Fig. 1. Simplified dendrogram showing SDS-PAGE clusters of 307 *Xanthomonas* strains, obtained by average linkage of correlation values. The number of strains per cluster is indicated within parentheses. *X.*, *Xanthomonas*; *X. c.*, *X. campestris*; *X. o.*, *X. oryzae*.

investigated here grouped together at $r = 0.90$. On the basis of DNA homology, *X. campestris* pv. *graminis* was found to be related to some other pathovars from grasses and cereals (Kerstens *et al.*, 1989).

The three *X. campestris* pv. *theicola* strains studied have a quite distinct protein profile. These pathogens of tea have only been found in a restricted area in Japan (Uehara & Arai, 1980), and few strains are available.

X. campestris pv. *melonis* yielded less well resolved protein profiles (Fig. 2), which explains the separation of these strains. Aberrant strains of other pathovars clustered around them, probably due to their diffuse and non-specific profiles.

X. campestris pv. *poinsetticola* appears to be very heterogeneous in protein profiles; nine strains were spread over three different clusters (3c, 7c and 19),

<i>X.c. pv. campestris</i> LMG 568T			1
<i>X.c. pv. juglandis</i> LMG 8045			2
<i>X.c. pv. phaseoli</i> LMG 8036	]	3a	3
<i>X.c. pv. phaseoli</i> LMG 7455			
<i>X.c. pv. cajani</i> LMG 558			
<i>X.c. pv. vignicola</i> LMG 839			
<i>X.c. pv. alfalfae</i> LMG 8019			
<i>X.c. pv. glycines</i> LMG 712			
<i>X.c. pv. phaseoli</i> LMG 830		3b	
<i>X.c. pv. alfalfae</i> LMG 8080	]	3c	
<i>X.c. pv. ricini</i> LMG 864			
<i>X.c. pv. vesicatoria</i> I LMG 910		3d	
<i>X.c. pv. vignicola</i> LMG 8752	]	3e	
<i>X.c. pv. dieffenbachiae</i> LMG 7399			
<i>X.c. pv. malvacearum</i> LMG 761		3f	
<i>X.c. pv. hyacinthi</i> LMG 739	]		4
<i>X.c. pv. hyacinthi</i> LMG 8042			
<i>X.c. pv. cassavae</i> LMG 673	]		5
<i>X.c. pv. cassavae</i> LMG 5246			
<i>X.c. pv. citri</i> LMG 682	]		6
<i>X.c. pv. vitians</i> LMG 937			
<i>X.c. pv. citri</i> LMG 8655			
<i>X.c. pv. juglandis</i> LMG 751		7a	7
<i>X.c. pv. vesicatoria</i> II LMG 911		7b	
<i>X.c. pv. pruni</i> LMG 852	]		
<i>X.c. pv. poinsettiiicola</i> LMG 5402		7c	
<i>X.c. pv. vitians</i> LMG 938		7d	
<i>X.c. pv. hederae</i> LMG 733		7e	
<i>X.c. pv. cassavae</i> LMG 5271			
<i>X.c. pv. phaseoli</i> LMG 7511			
<i>X.c. pv. dieffenbachiae</i> LMG 695			8
<i>X.c. pv. manihotis</i> LMG 784	]		9
<i>X.c. pv. manihotis</i> LMG 773			
<i>X.c. pv. cucurbitae</i> LMG 690			10
<i>X.c. pv. ricini</i> LMG 861			
<i>X.c. pv. begoniae</i> LMG 7303			
<i>X.c. pv. begoniae</i> LMG 552			11
<i>X.c. pv. pelargonii</i> LMG 7314			12
<i>X. populi</i> LMG 5743T			13
<i>X.o. pv. oryzae</i> LMG 5047T			14
<i>X.o. pv. oryzicola</i> LMG 797			15
<i>X.c. pv. theicola</i> LMG 8684			16
<i>X.c. pv. graminis</i> LMG 726			17
<i>X. axonopodis</i> LMG 538T			
<i>X. fragariae</i> LMG 708T			18
<i>X. albilineans</i> LMG 497			
<i>X.c. pv. pisi</i> LMG 847			
<i>X.c. pv. melonis</i> LMG 8670			19
<i>X. albilineans</i> LMG 494T			
<i>X. maltophilia</i> LMG 958T			

whereas four strains were unclustered. This heterogeneity suggests that *X. campestris* pv. *poinsetticola* comprises several biological entities. There is no correlation between the types found and the origin of the strains.

Within the main *X. campestris* group, comprising clusters 1 to 12, a number of homogeneous pathovars were delineated, i.e. *X. campestris* pv. *campestris* (cluster 1), pv. *hyacinthi* (cluster 4), pv. *cassavae* (cluster 5), pv. *citri* (cluster 6), pv. *manihotis* (cluster 9), pv. *cucurbitae* (cluster 10), pv. *begoniae* (cluster 11) and pv. *pelargonii* (cluster 12). Among these pathovars, *X. campestris* pv. *campestris* is very consistent by protein electrophoresis. Fifteen strains of diverse origin grouped at $r = 0.90$. It was previously found by Minsavage & Schaad (1983) that this pathovar is homogeneous on the basis of electrophoresis of membrane proteins. The uniformity of *X. campestris* pv. *campestris* despite its wide geographical distribution may be explained by the dissemination of this seedborne pathogen (Williams, 1980).

X. campestris pv. *hyacinthi* displayed highly characteristic protein profiles. The pathogen is virulent for *Hyacinthus orientalis* and has been isolated from nurseries (Kamerman, 1979). Although this pathovar is the oldest known xanthomonad (Wakker, 1883), it has been largely neglected in taxonomic studies, as its occurrence is restricted.

X. campestris pv. *manihotis* and *X. campestris* pv. *cassavae* are both pathogenic for cassava (*Manihot esculentum*), but cause different diseases. Previous comparisons between these two pathovars (Maraite & Weyns, 1979; Dos Santos & Dianese, 1985; Van den Mooter *et al.*, 1987b) have revealed that they can be differentiated both phenotypically and genotypically. In this study it is shown that both pathovars are distinct from other pathovars as well.

X. campestris pv. *citri* has been the subject of a number of taxonomic and epidemiological studies (Goto *et al.*, 1980; Hartung & Civerolo, 1987, 1989; Gabriel *et al.*, 1989). This pathovar is a collective designation for xanthomonads causing different diseases of citrus and related hosts (Civerolo, 1984). We have included strains of the most common form of citrus bacterial canker, the A group. The strains constituted a homogeneous cluster, a result consistent with those of RFLP analysis (Hartung & Civerolo, 1989). Two aberrant strains, LMG 8655 and LMG 8656, isolated from *Citrus aurantium* in Brazil, probably belong to group C of citrus bacterial canker disease (Namekata & Oliveira, 1972).

X. campestris pv. *cucurbitae* strains isolated from pumpkin (*Cucurbita maxima*) constituted a homogeneous group (cluster 10). The aberrant strain LMG 7479

(subcluster 3b) was isolated from cucumber (*Cucumis sativus*).

X. campestris pv. *begoniae* and *X. campestris* pv. *pelargonii* have been studied previously in more detail (Vauterin *et al.*, 1990a) and were characterized as uniform groups. In the present study representative strains of these pathovars were included to locate them in relation to other pathovars. Both pathovars display characteristic protein profiles.

A number of pathovars from leguminous plants (*Fabaceae*), namely *X. campestris* pv. *phaseoli* (including var. *fuscans*), pv. *alfalfae*, pv. *vignicola*, pv. *cajani* and pv. *glycines* were electrophoretically related to each other. They grouped in cluster 3, and mostly in subcluster 3a. It is interesting to note that some *X. campestris* pathovars, such as pv. *phaseoli*, pv. *glycines*, pv. *vignicola* and pv. *cajani*, have overlapping host ranges (Bradbury, 1984). Their phytopathological relatedness and the protein electrophoretic data suggest that the leguminous pathovars in cluster 3 are closely related. This relatedness has been confirmed by DNA-DNA hybridization (Hildebrand *et al.*, 1990; Vauterin *et al.*, 1990b). Strains of *X. campestris* pv. *alfalfae* and pv. *vignicola* showed great variability in their protein profiles, which was not correlated with the host or origin of the strains. The pathovar reference strain of *X. campestris* pv. *alfalfae*, LMG 497, was aberrant. The only leguminous pathovar studied so far which was unrelated to the main cluster 3 was *X. campestris* pv. *pisi*. However, only one strain of this pathovar is available.

A number of non-leguminous pathovars occurring in subclusters 3c to 3f were related to the leguminous group. These included strains of *X. campestris* pv. *ricini*, pv. *vesicatoria*, pv. *malvacearum*, pv. *poinsetticola* and pv. *dieffenbachiae*.

Most of the *X. campestris* pv. *ricini* strains formed a homogeneous group in subcluster 3c except the non-pathogenic pathovar reference strain LMG 861 and strains LMG 7441 and LMG 8682.

X. campestris pv. *vesicatoria* consists of two substantially different DNA homology groups (subgroup A and subgroup B; see Vauterin *et al.*, 1990b), represented in subcluster 3d and subcluster 7b. The latter group contained the pathovar reference strain LMG 911. No correlation was detected between the electrophoretic groupings and the diverse geographical origin or host plant (pepper or tomato). Two discrete subgroups of *X. campestris* pv. *vesicatoria* were also found by gas chromatographic profiling of fatty acids (unpublished data). A relationship between *X. campestris* pv. *vesicatoria* and the pathovars from crucifers (*Brassicaceae*) *X.*

Fig. 2. SDS-protein electrophoregrams of 50 representative *Xanthomonas* strains from the clusters obtained by numerical analysis. *X.*, *Xanthomonas*; *X. c.*, *X. campestris*; *X. o.*, *X. oryzae*. Note: strain LMG 497 should be named *X. c. alfalfae*.

campestris pv. *raphani* and *X. campestris* pv. *campestris*, suggested by Dye *et al.*, 1964, is unlikely on the basis of protein electrophoresis.

X. campestris pv. *malvacearum* constituted a fairly homogeneous electrophoretic group, although this worldwide pathogen of cotton consists of a number of pathological races (Brinkerhoff, 1970). The relatedness of *X. campestris* pv. *malvacearum* and *X. campestris* pv. *vesicatoria* subgroup A to xanthomonads from *Fabaceae* and to each other, as deduced from protein electrophoresis, is consistent with DNA-DNA hybridization data (Vauterin *et al.*, 1990b).

Another cluster of electrophoretically related pathovars was formed by some *X. campestris* pv. *juglandis*, pv. *vesicatoria* subgroup B, pv. *pruni*, pv. *vitians* and pv. *hederae*, grouping in cluster 7.

Among these, the protein profiles of *X. campestris* pv. *juglandis* (subcluster 7a) were very similar to *X. campestris* pv. *vesicatoria* subgroup B (subcluster 7b). Another type of this pathovar was found in cluster 2, whereas the pathovar reference strain LMG 747 displayed an aberrant protein profile.

SDS-protein profiles of *X. campestris* pv. *vitians* and *X. campestris* pv. *hederae* were highly similar (subclusters 7d and 7e), but pathovar reference strain *X. campestris* pv. *vitians* LMG 937 and strain LMG 8689 were aberrant. All *X. campestris* pv. *hederae* strains clustering in group 7e were isolated from *Hedera helix*. Two aberrant strains LMG 8667 and LMG 8668 were isolated from *Schefflera arboricola* and *Brassaia actinophylla*, respectively, and are clearly misnamed.

Another interesting case is represented by *X. campestris* pv. *dieffenbachiae*. Although only five strains from this pathovar were tested, the three strains in cluster 8 constitute a well-defined entity, including the pathovar reference strain LMG 695, all isolated from *Anthurium* in Brazil. The other two strains, isolated from *Dieffenbachia* in the USA, were closely related to *X. campestris* pv. *vignicola* and *X. campestris* pv. *malvacearum* in cluster 3e. It is very likely that the pathogens from *Anthurium* and *Dieffenbachia* constitute different biological entities.

In conclusion, it is shown here that a number of distinct protein electrophoretic types exist within *X. campestris*. Some of these groups correspond to described pathovars, others do not. The latter may include strains of different pathovars or may represent only part of a pathovar.

As the knowledge of the homogeneity of a *X. campestris* pathovar is important for both its classification and practical diagnosis, we have drawn particular attention to this aspect of the results. A number of pathovars are obviously homogeneous entities on the basis of protein electrophoresis: *X. campestris* pv. *campestris*, pv. *pelargonii*, pv. *manihotis*, pv. *cassavae*, pv.

hyacinthi, pv. *citri* (only the Asiatic form), pv. *pruni*, pv. *glycines* and pv. *graminis* Egli & Schmidt 1982. Others are heterogeneous as they display two or more discrete electrophoretic types. This was the case for *X. campestris* pv. *dieffenbachiae*, pv. *juglandis*, pv. *alfalfae*, pv. *poinsetticola*, pv. *vesicatoria* and pv. *vignicola*. In most of the latter four cases no correlation was observed between the electrophoretic groupings and the origin of the strains.

It is difficult to evaluate the homogeneity of a pathovar when only a limited number of strains are available, for instance *X. campestris* pv. *pisi*, pv. *cajani*, pv. *theicola*, or when a few strains are more or less aberrant (*X. campestris* pv. *begoniae*, pv. *cucurbitae*, pv. *malvacearum*, pv. *hederae* and pv. *melonis*). A problem also arises when the pathovar reference strains is clearly aberrant from the representative group, as was found for *X. campestris* pv. *ricini* and pv. *vitians*.

From the results presented here, it is clear that – although the taxonomy of *Xanthomonas* is not settled yet – the SDS-PAGE database of whole-cell protein patterns established in highly standardized conditions can be of great value to identify new and unknown *Xanthomonas* strains.

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