Taxonomy of xanthomonads from cereals and grasses based on SDS-PAGE of proteins, fatty acid analysis and DNA hybridization

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Strains of all *Xanthomonas* species, *X. campestris* pathovars pathogenic for cereals and grasses and strains of *X. campestris* pv. *campestris* were compared by SDS-PAGE of proteins, gas chromatography of fatty acid methyl esters and DNA–DNA hybridization. The groupings derived using all three methods correlated well with each other. Strains of *X. axonopodis* and *X. campestris* pv. *vasculorum* were heterogeneous, each comprising two groups (A and B); group A contained the type strain and pathovar reference strain, respectively. Besides the recognized *Xanthomonas* species *X. albilineans*, *X. campestris*, *X. fragariae*, *X. maltophilia*, *X. oryzae* and *X. populi*, the following five centres of variation could be delineated at DNA-binding levels of above 60%: (i) a group consisting of *X. campestris* pathovars *translucens*, *hordei*, *cerealis*, *secalis*, *undulosa* (the ‘*translucens* group’), *graminis*, *poae*, *arrhenatheri*, *phlei* (the ‘*graminis* group’) and *phleipratensis*; (ii) *X. axonopodis* type A together with *X. campestris* pv. *vasculorum* type A and *X. campestris* pv. *coracanae*; (iii) a group of *X. campestris* pv. *vasculorum* type B and *X. campestris* pv. *holcicola*; (iv) *Xanthomonas* strains isolated from *Bromus*; and (v) strains isolated from sugarcane in Guadeloupe. The type B strains of *X. axonopodis* appeared to be misclassified in *Xanthomonas*. An interim proposal for an improved classification of *Xanthomonas* is presented on the basis of the data.

Introduction

Many plant pathogens which were originally designated as separate *Xanthomonas* species were grouped into the single species *X. campestris* by Dye & Lelliott (1974) on the basis of their phenotypic uniformity. A special-purpose classification, distinguishing the former species as pathovars of *X. campestris*, was subsequently established (Dye *et al.*, 1980; Young *et al.*, 1978). However, with the development of taxonomic techniques such as electrophoresis of proteins and gas chromatography of fatty acids combined with computer-aided analysis of profiles, it became apparent that a number of phenotypes could be distinguished among strains called *X. campestris* (Stead, 1989; Vauterin *et al.*, 1990a, 1991a). A number of the phenotypically distinct *X. campestris* groups were found to correspond to groups revealed by DNA–DNA hybridization experiments (Hildebrand *et al.*, 1990; Vauterin *et al.*, 1990b). Interestingly, some of these groups contained virtually all of the pathovars from botanically related hosts. These included the pathovars from crucifers (*Brassicaceae*), the majority of pathovars

from leguminous hosts (*Fabaceae*), and a number of pathovars from cereals and grasses (family *Poaceae*) (Vauterin *et al.*, 1990b).

Although the xanthomonads pathogenic for cereals and grasses constitute an important group of pathovars, little is known about their natural relationships. The exceptions in this respect are *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, which cause bacterial blight and streak of rice, respectively (Ou, 1972). These important rice pathogens have been subject to a number of taxonomic and aetiological studies (for a review see Mew, 1987). *X. campestris* pvs. *oryzae* and *oryzicola* were recently reclassified as *X. oryzae* pathovars (Swings *et al.*, 1991).

X. campestris pv. *translucens* is pathogenic for a range of cereals including wheat, barley, rye and triticale. It is widespread in the USA, where it poses a problem in irrigated fields containing spring cereals (Schaad & Forster, 1985; Shane *et al.*, 1987). Several pathogenicity types were recognized (Boosalis, 1952; Hagborg, 1942), and these are now classified as pathovars of *X. campestris* (Dye *et al.*, 1980), or as *formae speciales* of *X. campestris* pv. *translucens*. Strains causing bacterial blight of barley (*Hordeum vulgare*) are contained in *X. campestris* pv. *hordei*, whereas strains causing black chaff of

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Abbreviation: FAME, fatty acid methyl ester.

wheat (*Triticum aestivum*) and triticale (*Tritico-secale* Whittmack) are classified as *X. campestris* pv. *undulosa*. Strains pathogenic for rye (*Secale cereale*) are designated *X. campestris* pv. *secalis*, whereas *X. campestris* pv. *cerealis* includes strains pathogenic for various cereals and the brome grass *Bromus inermis*. In this paper these pathovars together will be named the 'translucens group'. A clear definition of these pathovars in terms of phenotype, genotype or phytopathogenic specialization is not available. The significance of the different pathovars or *formae speciales* within the 'translucens group' is questionable. Rather than distinct pathotypes, a variable host range depending on the individual strain has been observed (Azad & Schaad, 1988; Cunfer & Scolari, 1982).

X. campestris pv. *graminis* is the causal agent of bacterial wilt of various grass genera, including economically important forage and pasture grasses. The disease has been reported in Europe, the USA and New Zealand (Egli & Schmidt, 1982; Roberts *et al.*, 1981; Van den Mooter *et al.*, 1987). The name *X. graminis* originally referred to all strains isolated from *Festuca*, *Lolium*, *Dactylis*, *Phleum* and *Arrhenatherum* (Egli *et al.*, 1975). However, Egli & Schmidt (1982) reported that the host range of some strains was restricted to either *Phleum*, *Arrhenatherum* or *Poa*. These strains were consequently renamed *X. campestris* pv. *phlei*, pv. *arrhenatheri* and pv. *poae*, respectively. The name *X. campestris* pv. *graminis* was retained for strains showing a broader host range including *Lolium*, *Festuca*, *Dactylis* and *Trisetum*. We will hereafter use the term 'graminis group' as a common name for these pathovars. Another pathovar, *X. campestris* pv. *phleipratensis*, causes leaf streak of *Phleum pratense*.

X. campestris pv. *holcicola* is the causal agent of bacterial streak disease on *Sorghum bicolor* and *S. halepense* (Elliott, 1930). Maize may also become infected after artificial inoculation (Leyns *et al.*, 1984; Bradbury, 1986). *Xanthomonas campestris* pv. *vasculorum* causes gumming disease (gummosis) on sugarcane (*Saccharum officinarum*). Its broad host range extends to a number of other *Poaceae*, including maize and sorghum, and even some palms (*Arecaceae*). Desai *et al.* (1965) described *X. campestris* pv. *coracanae* as a pathogen causing a vascular disease of ragi (*Eleusine coracana*) in India.

In addition to *X. oryzae* and the *X. campestris* pathovars, two other *Xanthomonas* species are known to affect members of the *Poaceae*: *X. albilineans* causes leaf scald of sugarcane (Ashby, 1929), whereas *X. axonopodis* causes vascular gummosis disease of *Axonopus scoparius* and *A. micay* grasses (Starr & Garcés, 1950).

Little is known about the phenotypic and genomic relatedness of the *X. campestris* pathovars which are

pathogenic for *Poaceae*. Van den Mooter *et al.* (1987) studied the four pathovars of grasses established by Egli & Schmidt (1982) and concluded that each of them constituted a distinct phenotypic entity. Stead (1989) used fatty acid fingerprints to investigate the relationships between all of the pathovars from cereals and grasses. This analysis revealed two major, related groups, corresponding with the pathovars of cereals from the 'translucens group' and the pathovars of grasses from the 'graminis group'.

We report here the results of a comparative study of 146 *Xanthomonas* strains from cereals, grasses and other *Poaceae*, using SDS-PAGE of proteins, gas chromatographic analysis of fatty acid methyl esters (FAMES), and DNA-DNA hybridization. The primary aim was to clarify the taxonomy and nomenclature of the species and pathovars of *Xanthomonas* which infect members of the family *Poaceae*.

Methods

Bacterial strains. A total of 146 strains representing all species of *Xanthomonas* and all *X. campestris* pathovars on members of the family *Poaceae* were studied. The strains used are listed in Table 1.

SDS-PAGE of whole-cell proteins. Culturing of cells, preparation and electrophoresis of protein samples and densitometric recording of gels were performed as described by Vauterin *et al.* (1991a). Densitometric records of gels were normalized by an iterative pattern-recognition algorithm (L. Vauterin & P. Vauterin, unpublished), using the GelCompar 1.3 software package (Helix CV, Biotechnology Business Development, Onafhankelijkheidslaan 38, B-9000 Gent, Belgium). The generation of a database of normalized traces and comparison of traces was done using the GelCompar 1.3 software. The correlation coefficient (*r*) was calculated between densitometric records, and cluster analysis was executed on the matrix of correlation values using the unweighted pair group method using arithmetic averages (UPGMA). The reproducibility of the electrophoresis technique was controlled (i) by preparing protein samples of all of the strains at least twice, (ii) by running most of the extracts in at least two gels, and (iii) by loading reference samples on each of the slab gels. The overall reproducibility of extraction and electrophoresis amounted to *r* = 0.92.

Gas chromatographic analysis of FAMES. Culturing of strains, extraction and quantitative analysis of FAMES using the Hewlett Packard model 5890A gas-liquid chromatograph were done as described by Vauterin *et al.* (1991b). FAME fingerprints were identified and compared using the Microbial Identification System (MIS) software package (version 3.2) provided by MIDI (Microbial ID Inc., Newark, DE, USA). The Euclidean distance coefficient was calculated between profiles and clustering was achieved using UPGMA. In each of the gas chromatography runs, a calibration mixture of known standards (MIDI) and *X. maltophilia* strain LMG 958 were included to test the reproducibility. The variation detected among FAME profiles of different runs of strain LMG 958 was about 2 Euclidean distance units. FAME clusters were subjectively delineated at about 10 units of Euclidean distance. The mean percentages and standard deviations were calculated for each cluster. Fatty acids for which the product of the mean percentage times the percentage of samples containing the fatty acid was less than 0.25 were excluded.

Table 1. Source and group assignment of the bacterial strains

Species and/or pathovar	Group based on†:		
	Protein profile	FAMEs	DNA-DNA homology
<i>X. albilineans</i>			
LMG 482 (NCPPB 887)†, LMG 487 (NCPPB 1050), LMG 488 (NCPPB 1244)	13	16	12
LMG 490 (NCPPB 2247), LMG 494 ^T (NCPPB 2969)†	13	16	12
Strains from Guadeloupe received as <i>X. albilineans</i>			
LMG 471†, LMG 473, LMG 476†	6	9	7
<i>X. axonopodis</i>			
LMG 537 (ICMP 74)†	10	18	u
LMG 541 (ICMP 4697)†	10	18	9
LMG 542 (ICMP 4698)†	–	18	9
LMG 538 ^T (NCPPB 457)†, LMG 539 (NCPPB 2375)†, LMG 540 (NCPPB 2376)	4	13	3
<i>X. campestris</i> pv. <i>arrhenatheri</i>			
LMG 588 (E705 T. Egli)†, LMG 590 (E711 T. Egli), LMG 591 (E720 T. Egli)	1	2	1
LMG 592 (E730 T. Egli), LMG 727* (E719 T. Egli)†, LMG 7384 (NCPPB 2699)	1	2	1
<i>X. campestris</i> pv. <i>campestris</i>			
LMG 568 ^T (NCPPB 528)†, LMG 583 (HMB 142)†, LMG 8032 (CFBP 1124)†	9	6	8
<i>X. campestris</i> pv. <i>cerealis</i>			
LMG 679* (NCPPB 1944)†, LMG 880 (NCPPB 2817)†, LMG 881 (NCPPB 2819)	1	3	1
LMG 887 (NCPPB 2827), LMG 890 (NCPPB 2830), LMG 7392 (NCPPB 1836)	1	3	–
LMG 7393 (NCPPB 1943)	1	3	–
LMG 891 (NCPPB 2831)	1	4	–
<i>X. campestris</i> pv. <i>coracanae</i>			
LMG 686* (NCPPB 1786)†, LMG 7476 (ICMP 2103)†	5	5	4
<i>X. campestris</i> pv. <i>graminis</i>			
LMG 587 (IPO 118), LMG 593 (E733 T. Egli), LMG 595 (E741 T. Egli)	2	1	–
LMG 598 (Xg81-8 F. Leyns), LMG 600 (Xg81-16 F. Leyns)	2	1	–
LMG 607 (Xg81-28 F. Leyns), LMG 622 (Xg81-54 F. Leyns)	2	1	–
LMG 623 (Xg81-56 F. Leyns), LMG 626 (Xg81-61 F. Leyns)	2	1	–
LMG 713 (218 M. Van den Mooter)†, LMG 726* (NCPPB 2700)†	2	1	1
LMG 7405 (NCPPB 2695), LMG 7406 (NCPPB 2696), LMG 7407 (NCPPB 2689)	2	1	–
LMG 7408 (NCPPB 2701)	2	1	–
<i>Bromus</i> isolates received as <i>X. campestris</i> pv. <i>graminis</i>			
LMG 8267 (ICMP 7419), LMG 8268 (ICMP 6436), LMG 8269 (ICMP 6078)†	7	15	6
LMG 8270 (ICMP 6054), LMG 8271 (ICMP 6435), LMG 8272 (ICMP 6438)†	7	15	6
LMG 8273 (ICMP 6482)	7	15	–
<i>X. campestris</i> pv. <i>holcicola</i>			
LMG 736* (NCPPB 2389)†, LMG 7416 (NCPPB 989)†, LMG 7417 (NCPPB 1060)	8	12	5
LMG 7418 (NCPPB 1241), LMG 7489 (ICMP 3489)†, LMG 8276 (ICMP 3104)	8	12	5
LMG 8277 (ICMP 3409)	8	12	–
<i>X. campestris</i> pv. <i>hordei</i>			
LMG 737* (NCPPB 2389)†, LMG 884 (NCPPB 2824), LMG 889 (NCPPB 2829)	1	4	1
LMG 8279 (ICMP 1670)†, LMG 8280 (ICMP 1671)	1	4	1
LMG 879 (NCPPB 2181)†, LMG 882 (NCPPB 2820)†, LMG 8278 (ICMP 295)	1	3	1
LMG 8281 (ICMP 3100)	1	3	–
<i>X. campestris</i> pv. <i>phlei</i>			
LMG 716 (221 M. Van den Mooter)†, LMG 717 (222 M. Van den Mooter)	1	2	1
LMG 718 (224 M. Van den Mooter), LMG 719 (227 M. Van den Mooter)	1	2	–
LMG 720 (228 M. Van den Mooter), LMG 723 (231 M. Van den Mooter)	1	1	–
LMG 730* (E729 T. Egli)†	1	3	1
<i>X. campestris</i> pv. <i>phleipratensis</i>			
LMG 843* (NCPPB 1837)†	1	1	1
<i>X. campestris</i> pv. <i>poae</i>			
LMG 594 (E735 T. Egli)†, LMG 728* (E724 T. Egli)†	1	1	1
<i>X. campestris</i> pv. <i>secalis</i>			
LMG 877 (NCPPB 974)	1	u	–
LMG 883* (NCPPB 2822)†, LMG 7507 (ICMP 292)†	1	3	1
<i>X. campestris</i> pv. <i>translucens</i>			
LMG 875 (ICPB XT7), LMG 878 (NCPPB 1942), LMG 5259 (Xt102 B. Cunfer)†	1	3	1
LMG 5260 (Xt110 B. Cunfer)†, LMG 5261 (Xt108 B. Cunfer)	1	3	1
LMG 5262 (Xt110 B. Cunfer), LMG 5263 (Xt116 B. Cunfer)	1	3	–
LMG 876* (NCPPB 973)†	1	4	1

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Table 1 – *continued*

Species and/or pathovar	Group based on†:		
	Protein profile	FAMEs	DNA–DNA homology
<i>X. campestris</i> pv. <i>undulosa</i>			
LMG 885 (NCPPB 2825)†, LMG 886 (NCPPB 2826), LMG 888 (NCPPB 2828)†	1	3	1
LMG 892* (NCPPB 2821)†, LMG 8283 (ICMP 282)	1	3	1
LMG 8282 (ICMP 297)	1	4	–
<i>X. campestris</i> pv. <i>vasculorum</i>			
LMG 894 (ICPB XV24), LMG 895 (NCPPB 181)†, LMG 897 (NCPPB 184)	4	14	3
LMG 898 (NCPPB 185), LMG 901* (NCPPB 796)†	4	14	3
LMG 899 (NCPPB 186)†, LMG 903 (NCPPB 891)†	4	1	3
LMG 900 (NCPPB 795)†, LMG 902 (NCPPB 889)†, LMG 8284 (ICMP 326)†	8	12	5
LMG 8285 (ICMP 253)†	6	u	u
<i>X. fragariae</i>			
LMG 704 (ICMP 3304), LMG 706 (F1 Benaki)†, LMG 707 (F8 Benaki)	11	7	10
LMG 708 ^T (ICMP 1469)†, LMG 709 (ICMP 1822)	11	7	10
<i>X. maltophilia</i>			
LMG 957 (ATCC 17448)†, LMG 958 ^T (ATCC 13637)†	u	8	u
<i>X. oryzae</i> pv. <i>oryzae</i>			
LMG 634 (CIAT 1185), LMG 635 (CIAT 1186), LMG 643 (PXO61 T. W. Mew)	3a	10	–
LMG 645 (T7133 Wakimoto), LMG 646 (T7147 Wakimoto)	3a	10	–
LMG 647 (T7174 Wakimoto), LMG 650 (H75304 Wakimoto)	3a	10	–
LMG 795 (NCPPB 1153)†, LMG 803 (PXO71 T. W. Mew)	3a	10	2
LMG 804 (PXO79 T. W. Mew), LMG 806 (PXO86 T. M. Mew)	3a	10	–
LMG 5047 ^T (ICMP 3125)†, LMG 6518 (IRN 235 IRRI)†	3a	10	2
<i>X. oryzae</i> pv. <i>oryzicola</i>			
LMG 654 (ICMP 1621), LMG 656 (ICMP 3129), LMG 657 (ICMP 3130)	3b	11	–
LMG 658 (ICMP 3131), LMG 660 (ICMP 3133), LMG 661 (ICMP 3134)	3b	11	–
LMG 665 (ICMP 4645)†, LMG 792 (NCPPB 1150), LMG 793 (NCPPB 11581)†	3b	11	2
LMG 797* (NCPPB 15685)†, LMG 798 (NCPPB 1632)	3b	11	2
<i>X. populi</i>			
LMG 974 (PC3 M. Ridé)†, LMG 975 (S8 M. Ridé), LMG 5743 ^T (CNBP 1817)†	12	17	11
LMG 5753 (CNBP 1832)†, LMG 5755 (CNBP 2226)	12	17	11
<i>X. campestris</i> from <i>Bromus</i> (without pathovar designation)			
LMG 947 (CNBP 1976)†	8	u	6

Abbreviations and symbols for strains: ATCC, American Type Culture Collection, Rockville, MD, USA; CFBP, Collection Française de Bactéries Phytopathogènes, Paris, France; CIAT, Rhizobium Collection, Centro Internacional de Agricultura Tropical, Cali, Colombia; CNBP, Collection Nationale de Bactéries Phytopathogènes, Angers, France; HMB, Collection of H. Maraité, Louvain-la-Neuve, Belgium; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; ICPB, International Collection of Phytopathogenic Bacteria, Davis, CA, USA; IPO, Instituut voor Plantenziektenkundig Onderzoek, Wageningen, The Netherlands; IRRI, International Rice Research Institute, Los Baños, The Philippines; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent; NCPPB, National Collection of Plant Pathogenic Bacteria, Hapenden, UK. A superscript T denotes a type strain, and an asterisk (*) a pathovar reference strain. † denotes strains which have been tested by DNA–DNA hybridization.

† –, Not tested.; u, unclustered or ungrouped strain.

DNA hybridization. DNA was extracted from 64 strains representative of the protein and FAME clusters. High-molecular-mass DNA was purified using the method of Marmur (1961). DNA-binding measurements (%D) were obtained using the spectrophotometric method described by De Ley *et al.* (1970). The renaturation rates were measured in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl plus 0.015 M-trisodium citrate, pH 7.0) at the average optimal renaturation temperature of 80.8 °C, which was calculated from the mol% G + C (De Ley, 1970). Each experiment was repeated at least once. The overall experimental error of the method, which was calculated from the repeats, averaged $\pm 6\%$. In order to reduce the size of the matrix of %D values, uniform groups were delineated above 60% and the internal mean %D value together with its standard deviation was calculated for each group. Sets of %D values between these groups were averaged in the same way.

Results

SDS-PAGE protein patterns

An abridged dendrogram, with homogeneous groups represented as triangles, is shown in Fig. 1. Protein clusters were delineated above $r = 0.80$, except for cluster 13 (*X. albilineans*), which was delineated at $r = 0.74$.

All of the strains of *X. campestris* pvs. *poae*, *phlei* and *arrhenatheri* (i.e. the 'graminis group'), all of the strains of *X. campestris* pvs. *translucens*, *hordei*, *undulosa*, *secalis* and *cerealis* (i.e. the 'translucens group'), and the single

Fig. 1. Simplified dendrogram obtained by average linkage of correlation values between SDS-PAGE protein patterns of the xanthomonads studied. The number of strains per cluster is given in parentheses. *X.*, *Xanthomonas*; *X. c.*, *X. campestris*; *X. o.*, *X. oryzae*.

strain of *X. campestris* pv. *phleipratensis* LMG 843 grouped together in cluster 1. Each of the pathovars *phlei*, *poae* and *arrhenatheri* could be distinguished as a homogeneous subgroup within cluster 1 (data not shown). *X. campestris* pv. *graminis* (Egli & Schmidt, 1982) constituted a distinct cluster 2. All of the *X. oryzae* strains grouped in cluster 3. This cluster could be subdivided into two subclusters (3a and 3b), which

corresponded to *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. Seven of the *X. campestris* pv. *vasculorum* strains, including the pathovar reference strain LMG 901 (designated *X. campestris* pv. *vasculorum* type A) were recovered in cluster 4 along with three *X. axonopodis* strains, including the type strain LMG 538 (*X. axonopodis* type A). The two strains of *X. campestris* pv. *coracanae* constituted a separate protein electrophoretic type (cluster 5). Three strains received as *X. albilineans* from Guadeloupe (designated Guad strains) and one strain of *X. campestris* pv. *vasculorum* LMG 8285 from India formed a distinct cluster (cluster 6). Seven strains received as *X. campestris* pv. *graminis* which were isolated from *Bromus willdenowii* in New Zealand grouped together with *X. campestris* LMG 947 (no pathovar name), which was isolated from *Bromus carinatus* in France (cluster 7). A second group of *X. campestris* pv. *vasculorum*, comprising three strains (type B) was indistinguishable from *X. campestris* pv. *holcicola* (cluster 8). Three strains of *X. campestris* pv. *campestris*, including the type strain LMG 568, were clearly separated from all other pathovars (cluster 9). Cluster 10 comprised a second protein type of *X. axonopodis* (type B), and contained two weakly related strains, which differed strongly from the other *X. axonopodis* strains. The species *X. fragariae*, *X. populi* and *X. albilineans* were represented by clusters 11, 12 and 13, respectively. *X. albilineans* was heterogeneous on the basis of numerical analysis ($r = 0.74$). This was due to the presence of a variable heavy protein band in the 36 kDa molecular mass zone. Two strains of *X. maltophilia* were only distantly related to the other xanthomonads ($r = 0.48$).

Fatty acid profiles

An abridged dendrogram obtained by UPGMA analysis of the whole-cell FAME patterns for 146 strains is shown in Fig. 2. Eighteen FAME clusters were delineated at 10 Euclidean distance units while three strains remained unclustered. The fatty acid comparison within each of the groups is presented in Table 2.

The species *X. fragariae* (FAME cluster 7), *X. albilineans* (FAME cluster 16) and *X. populi* (FAME cluster 17) were homogeneous and clearly separated from each other. The fatty acid profile of *X. maltophilia* (FAME cluster 8) showed a high similarity to *X. fragariae*. However, *X. maltophilia* could be easily characterized by the presence of a 17:0 cyclopropane fatty acid, which was absent in all the other xanthomonads. Strains of *X. oryzae* formed two distinct and homogeneous groups, which corresponded to *X. oryzae* pv. *oryzae* (FAME cluster 10) and *X. oryzae* pv. *oryzicola* (FAME cluster 11). As observed by protein analysis,

Fig. 2. Simplified dendrogram obtained by average linkage of Euclidean distance values between FAME profiles of the xanthomonads studied. The number of strains per cluster is given in parentheses. *X.*, *Xanthomonas*; *X. c.*, *X. campestris*; *X. o.*, *X. oryzae*.

X. axonopodis consisted of two different types. FAME cluster 13 corresponded with type A as defined in the protein analysis, and contained the type strain. The type B strains, represented in FAME cluster 18, differed markedly from all the other xanthomonads studied, as they lacked a number of fatty acids typical of the genus *Xanthomonas*, for example *iso*-11:0, *iso*-3OH-11:0, *iso*-3OH-13:0, *iso*-15:0 and *iso*-17:0, whereas they produced unusually large amounts of unresolved 18:1 fatty

acids termed 'summed feature 7' (see Table 2). The Guad strains received as *X. albilineans* had a highly characteristic and uniform FAME profile (FAME cluster 9).

X. campestris pvs. *graminis* and *poae* occurred in cluster 1 together with *X. campestris* pv. *phleipratensis*, whereas the other pathovars from grasses, *X. campestris* pvs. *phlei* and *arrhenatheri*, were found in cluster 2. Most of the strains of the pathovars from the 'translucens group' (i.e. *X. campestris* pvs. *cerealis*, *hordei*, *secalis*, *translucens* and

Table 2. Major fatty acids (mean percentage of total) of 18 FAME clusters of the xanthomonads used in this study

Fatty acid	FAME cluster:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
10:0	0.5	0.6	0.4	0.7	1.1	0.4	0.9	0.7	0.9	2.2	0.7	0.8	0.5	—	—	0.5	1.7	—
iso-11:0	5.7	6.4	5.7	4.9	4.9	4.3	5.6	4.3	4.1	5.0	4.4	3.0	3.4	5.0	6.1	5.2	3.6	—
3OH-10:0	—	—	—	—	0.9	—	—	—	—	0.7	—	—	—	—	—	—	0.6	3.1
Unknown 11:798*	—	—	—	—	1.8	1.7	0.6	2.0	—	1.6	1.9	1.6	2.2	1.2	1.6	—	1.0	—
12:0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4.3
iso-3OH-11:0	3.3	3.9	2.9	2.4	4.3	2.9	4.7	2.0	0.9	3.0	2.6	2.0	1.7	2.0	3.8	2.8	2.1	—
3OH-11:0	—	—	—	—	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—
iso-13:0	0.8	2.4	0.5	—	—	—	1.8	0.6	0.5	—	—	—	—	—	—	—	1.4	—
iso-3OH-12:0	—	1.0	1.1	1.0	0.6	—	—	0.3	2.9	—	—	—	—	—	—	—	—	—
2OH-12:0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4.2
3OH-12:0	1.6	1.7	1.4	1.5	5.6	2.3	3.7	3.1	2.2	4.7	1.2	2.7	3.1	1.2	2.0	0.8	3.7	4.4
iso-14:0	1.2	4.7	3.8	3.5	0.4	—	—	0.9	14.4	—	—	—	—	—	—	—	—	—
14:0	0.8	3.3	1.9	2.7	1.1	0.6	2.9	2.9	1.0	—	0.8	1.9	0.7	—	1.3	—	5.4	0.4
iso-3OH-13:0	3.2	3.3	2.2	1.9	7.8	4.9	4.3	4.3	1.0	3.8	5.9	2.8	4.3	6.1	5.5	2.3	3.2	—
2OH-13:0	—	—	—	—	0.5	0.5	0.6	0.8	0.3	—	—	—	—	—	—	—	—	—
iso-15:1 F†	0.5	2.6	1.0	0.9	—	0.3	—	0.7	0.5	—	—	—	—	—	—	—	—	—
iso-15:1 G†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.4	—
iso-15:0	33.5	31.0	27.5	21.2	19.9	28.1	39.5	34.6	17.4	3.2	10.7	16.1	20.0	24.6	21.3	1.9	23.5	—
anteiso-15:0	4.0	5.9	6.1	5.4	7.8	15.2	10.5	15.9	5.5	—	1.6	2.9	0.6	1.4	2.7	—	4.7	—
15:1 A†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.9	—
15:1 B†	—	0.4	0.5	0.5	1.1	0.9	—	—	0.5	—	—	—	—	—	—	—	—	—
15:0	—	0.6	0.6	0.9	1.6	1.6	1.4	0.5	0.4	—	—	2.1	1.5	1.3	2.0	—	3.5	—
iso-16:0	2.1	1.9	7.0	6.6	2.0	1.8	0.5	1.6	18.4	—	—	1.4	—	—	1.7	4.2	—	—
16:1 B†	—	1.6	—	—	2.0	0.4	—	1.9	1.1	2.7	0.8	—	1.3	—	0.7	—	17.3	—
16:1 cis 9	20.0	16.8	21.5	26.7	17.2	11.3	11.4	7.6	14.9	26.8	23.3	24.4	19.6	9.9	8.9	11.4	—	19.8
16:1 C†	—	—	—	—	—	—	0.6	—	—	—	—	—	—	—	—	—	1.1	—
16:0	3.2	3.7	4.3	7.7	5.0	3.1	3.3	5.0	4.3	20.6	13.9	15.9	9.1	2.5	4.9	13.4	9.2	20.4
iso-17:1 F†	10.1	4.2	7.0	6.2	4.3	9.3	2.8	4.2	2.1	7.6	13.6	11.9	8.4	21.7	25.7	27.5	—	—
iso-17:0	7.4	2.1	3.1	3.3	5.5	7.2	2.9	2.5	2.5	13.0	16.1	6.8	15.1	20.5	11.0	21.3	3.0	—
anteiso-17:0	—	—	—	—	0.5	1.0	—	0.4	—	—	0.5	1.0	—	—	—	—	—	—
17:1 B†	0.5	—	0.4	0.5	1.6	1.8	—	—	0.3	—	—	1.3	2.2	1.9	0.5	—	2.9	—
17:0 cyclopropane	—	—	—	—	—	—	—	1.3	—	—	—	—	—	—	—	—	—	—
iso-18:0	—	—	—	—	—	—	—	—	0.5	—	—	—	—	—	—	—	—	0.6
18:1 cis 9	0.6	—	—	—	0.5	—	—	0.8	0.4	2.4	0.9	0.6	3.0	—	—	—	7.8	—
18:1 B†	—	0.6	0.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
iso-19:0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.2	—	—
19:0 cyclopropane 11-12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2.7
Summed feature 7‡	—	—	—	—	—	—	—	0.5	0.3	1.8	—	—	4.0	—	—	2.0	—	40.0

* The identity of the fatty acid which has an equivalent chain length of 11.798 is unknown.

† The double bond position indicated by the capital letters is unknown.

‡ Fatty acids 18:1 cis 11, 18:1 trans 9 and 18:1 trans 6 could not be separated from each other by gas chromatography using the MIS system and were named together summed feature 7.

undulosa) grouped in cluster 3. Clusters 1 to 3 were closely related to each other at a level of 12 Euclidean distance units. Five strains of *X. campestris* pv. *hordei* and one strain each of *X. campestris* pv. *cerealis*, pv. *translucens* and pv. *undulosa* formed FAME cluster 4 and differed from clusters 1 to 3 mainly in that they produced a lower amount of the fatty acid iso-15:0 and a higher amount of 16:1 cis 9. The *Bromus willdenowii* isolates from New Zealand, received as *X. campestris* pv. *graminis*, were different from the other *X. campestris* pv. *graminis* strains and clustered in FAME cluster 15. The French *Bromus carinata* isolate LMG 947 was closely related to cluster 15. Within *X. campestris* pv. *vasculorum*, the same types were found as revealed by protein electrophoresis.

Strains corresponding to type A were contained in FAME cluster 14, except for LMG 899 and LMG 903, which showed an overall FAME profile similar to *X. campestris* pv. *graminis* (cluster 1). Type B was represented in FAME cluster 12 together with *X. campestris* pv. *holcicola*. *Xanthomonas campestris* pv. *coracanae* had a distinct fatty acid profile (cluster 5). The strains of *X. campestris* pv. *campestris* were separated from all other pathovars (cluster 6).

DNA hybridization

A total of 240 DNA binding values were measured among the 64 selected strains (see Table 1). Twelve DNA

Fig. 3. Schematized DNA homology matrix showing the averaged %D values within and between DNA homology groups delineated at 60% D.

homology groups were distinguished (Fig. 3). Average %D values with corresponding standard deviations for the *X. campestris* pathovars are given in Fig. 4. The groupings based on DNA homology were consistent with the clustering obtained by protein electrophoresis and FAME analysis. The species *X. albilineans*, *X. fragariae*, *X. oryzae* and *X. populi* each constituted a homogeneous DNA homology group (nos 12, 10, 2 and 11, respectively). Within *X. oryzae*, no differentiation could be made between the pathovars *oryzae* and *oryzicola*. The two *X. maltophilia* strains showed a rather low DNA homology with each other and were distinct from all the other xanthomonads. The existence of two types by protein electrophoresis within *X. axonopodis* and *X. campestris* pv. *vasculorum* was confirmed by DNA

hybridization. *X. axonopodis* type A was related to *X. campestris* pv. *vasculorum* type A as they were both found in DNA homology group 3. *X. axonopodis* type B strain LMG 537 was only loosely related to the other *X. axonopodis* strains of type B (DNA homology group 9). *X. campestris* pv. *vasculorum* type B shared high DNA binding with *X. campestris* pv. *holcicola* (DNA homology group 5). *X. campestris* pv. *coracanae* (DNA homology group 4) was related to *X. campestris* pv. *vasculorum* type A and *X. axonopodis* type A (DNA homology group 3). In agreement with the results of protein electrophoresis and FAME analysis, the *Bromus willdenowii* isolates from New Zealand were closely related to the French isolate LMG 947 from *Bromus carinatus* (DNA homology group 6). The Guadeloupe strains constituted a separate group

DNA homology group									
<i>X. c. translucens</i> - <i>graminis</i> group	1	78% <i>s</i> = 11% <i>n</i> = 48							
<i>X. oryzae</i>	2	12% <i>s</i> = 3% <i>n</i> = 7	91% <i>s</i> = 7% <i>n</i> = 6						
<i>X. c. vasculorum</i> A <i>X. axonopodis</i> A	3	17% <i>s</i> = 13% <i>n</i> = 2	42% <i>s</i> = 8% <i>n</i> = 4	90% <i>s</i> = 6% <i>n</i> = 6					
<i>X. c. coracanae</i>	4	18% <i>s</i> = 6% <i>n</i> = 2	30% <i>s</i> = 3% <i>n</i> = 3	59% <i>s</i> = 3% <i>n</i> = 3	84%				
<i>X. c. holcicola</i> <i>X. c. vasculorum</i> B	5	8% <i>s</i> = 9% <i>n</i> = 3	48% <i>s</i> = 5% <i>n</i> = 3	38% <i>s</i> = 6% <i>n</i> = 5	39% <i>s</i> = 7% <i>n</i> = 2	90% <i>s</i> = 4% <i>n</i> = 6			
<i>Bromus</i> isolates	6	25% <i>s</i> = 1% <i>n</i> = 2	38% <i>s</i> = 4% <i>n</i> = 2	41% <i>s</i> = 5% <i>n</i> = 3	41% <i>s</i> = 5% <i>n</i> = 3	38% <i>s</i> = 6% <i>n</i> = 2	88% <i>s</i> = 6% <i>n</i> = 2		
Guad strains	7	30% <i>s</i> = 13% <i>n</i> = 3	25% <i>s</i> = 4% <i>n</i> = 2	12% <i>s</i> = 3% <i>n</i> = 2	18% <i>s</i> = 4% <i>n</i> = 2	7% <i>s</i> = 3% <i>n</i> = 2	31% <i>s</i> = 4% <i>n</i> = 2	98%	
LMG 8285		40% <i>s</i> = 11% <i>n</i> = 3	21% <i>s</i> = 4% <i>n</i> = 2	13% <i>s</i> = 3% <i>n</i> = 2	14% <i>s</i> = 4% <i>n</i> = 2	20% <i>s</i> = 4% <i>n</i> = 2	29% <i>s</i> = 3% <i>n</i> = 2	52% <i>s</i> = 3% <i>n</i> = 2	100%
<i>X. c. campestris</i>	8	18% <i>s</i> = 4% <i>n</i> = 4	15% <i>s</i> = 11% <i>n</i> = 2	17% <i>s</i> = 2% <i>n</i> = 3	29% <i>s</i> = 4% <i>n</i> = 2	14% <i>s</i> = 3% <i>n</i> = 2	20% <i>s</i> = 4% <i>n</i> = 2	32% <i>s</i> = 3% <i>n</i> = 2	3% <i>s</i> = 2% <i>n</i> = 3
		1	2	3	4	5	6	7	LMG 8285
									8

Fig. 4. Averaged %*D* values and corresponding standard deviations within and between DNA homology groups 1 to 8. *s*, standard deviation; *n*, number of values.

(DNA homology group 7), with the Indian strain LMG 8285, received as *X. campestris* pv. *vasculorum*, being related to it at 52%.

Discussion

The pathovars of the '*translucens* group' comprise a major phenotypic group of *X. campestris* pathovars from *Poaceae*. On the basis of the present data, no distinction can be made between the members of this group, i.e. *X. campestris* pvs. *translucens*, *hordei*, *cerealis*, *secalis* and *undulosa*. Furthermore, these pathovars cannot be clearly defined on the basis of pathogenicity (Cunfer & Scolari, 1982). For example, it has been shown for *X. campestris* pv. *translucens* strains with a broad host range, that the pathogenicity towards certain hosts may be inactivated by single point mutations (Mellano & Cooksey, 1988). Our results clearly support previous data on pathogenicity, serological relationships and fatty acid composition (Azad & Schaad, 1988; Stead, 1989), which suggested

that the subdivision of *X. campestris* into pvs. *translucens*, *hordei*, *cerealis*, *secalis* and *undulosa* (Dye *et al.*, 1980) was not justified.

Another interesting case was represented by the pathovars of the '*graminis* group'. The protein patterns for *X. campestris* pvs. *arrhenatheri*, *phlei* and *poae* were very similar to those for the '*translucens* group'. In contrast, *X. campestris* pv. *graminis* had a different protein pattern. The classification of these strains as a separate pathovar of *X. campestris* (Egli & Schmidt, 1982) seems to be justified on the basis of the present data. The FAME profiles of all of the pathovars of the '*graminis* group', including *X. campestris* pv. *graminis*, were similar to those of the '*translucens* group' (FAME clusters 1 to 3). However, it was possible to distinguish the individual pathovars *graminis*, *arrhenatheri* and *phlei* as each constituted a homogeneous subgroup. The single strain of *X. campestris* pv. *phleipratensis* was clearly distinguishable from *X. campestris* pv. *phlei* on the basis of protein and FAME profiles. This strain causes leaf streak on *Phleum pratense*, whereas strains of

X. campestris pv. *phlei* cause bacterial wilt. DNA hybridization data revealed that all pathovars of both the 'translucens group' and the 'graminis group' were inter-related at 78% *D*, which indicates that they all belong to the same species. The seven *Bromus* isolates, received as *X. campestris* pv. *graminis*, which were isolated in New Zealand, together with *Bromus* isolate LMG 947 from France were not related to *X. campestris* pv. *graminis* nor to any of the other species. These strains therefore constitute a new species of *Xanthomonas*.

Another distinct group of organisms comprised the rice pathogens *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Both pathovars displayed characteristic protein and fatty acid profiles. In contrast, the DNA homology data did not reveal any difference between pathovars *oryzae* and *oryzicola*. The high genomic relatedness of the two rice pathogens (91% *D*), together with their distinct position in relation to the different *Xanthomonas* taxa, agrees with their recent reclassification as *X. oryzae* (Swings *et al.*, 1991). However, the different symptoms produced and the distinct phenotypes of the two groups support the retention of the pathovar names *oryzae* and *oryzicola* (Swings *et al.*, 1991).

A more complex situation was found in *X. axonopodis* and *X. campestris* pv. *vasculorum*. *X. axonopodis* consisted of two substantially different groups (types A and B). Type A contained the original isolates from Colombia (Starr & Garcés, 1950) and included the type strain LMG 538. Based on fatty acid analysis, *X. axonopodis* type B strains were not xanthomonads, as they produced a markedly different fatty acid profile from the other strains. In fact, according to the MIS library they resemble the profile of *Pseudomonas aeruginosa*. *X. campestris* pv. *vasculorum* also consisted of two types (A and B); type A strains appeared to be similar to *X. axonopodis* type A strains by protein electrophoresis and DNA hybridization, although their fatty acid profiles were different. Based on DNA hybridization, homology group 3, consisting of *X. axonopodis* type A and *X. campestris* pv. *vasculorum* type A, was related to *X. campestris* pv. *coracanae* (DNA homology group 4) at 59% *D* (Fig. 4). A more distant relationship was also observed between these groups and DNA homology groups 2 (*X. oryzae*), 5 (*X. campestris* pvs. *holcicola* and *vasculorum* type B) and 6 (*Bromus* isolates). *X. campestris* pv. *vasculorum* type B was indistinguishable from *X. campestris* pv. *holcicola* by all three methods. The Indian isolate from sugarcane, LMG 8285, received as *X. campestris* pv. *vasculorum*, was not related to *X. campestris* pv. *vasculorum* type A (DNA homology group 3) or B (DNA homology group 5). Based on DNA homology, this strain was related at 52% to the Guad strains received as *X. albilineans* from sugarcane in Guadeloupe

(DNA homology group 7). The latter strains showed no relationship to *X. albilineans* or to any of the other xanthomonads studied.

In conclusion, protein electrophoresis, fatty acid analysis, and DNA hybridization were shown to yield virtually identical groupings of the xanthomonads studied. One of the major findings of this study was that many of the *Xanthomonas* species and pathovars grouped into taxa which do not reflect the current classification of that genus. As discussed previously (Vauterin *et al.*, 1990b; 1991b), the DNA homology groups should be the framework for a revised classification of the genus *Xanthomonas*. None of the DNA homology groups recovered in the present investigation showed any relationship with previously defined *Xanthomonas* DNA homology groups (Vauterin *et al.*, 1990b). A future classification of the *Xanthomonas* taxa delineated here might be as follows.

(1) *Xanthomonas oryzae*, consisting of pvs. *oryzae* and *oryzicola*;

(2) *Xanthomonas translucens*, to include all *X. campestris* pathovars of the 'translucens group', i.e. pvs. *translucens*, *hordei*, *cerealis*, *undulosa* and *secalis* (Dye *et al.*, 1980), and the pathovars of the 'graminis group', i.e. *X. campestris* pvs. *graminis*, *poae*, *phlei* and *arrhenatheri* (Egli & Schmidt, 1982), together with *X. campestris* pv. *phleipratensis*;

(3) *Xanthomonas vasculorum*, consisting of *X. axonopodis* A and *X. campestris* pv. *vasculorum* A together with *X. campestris* pv. *coracanae*;

(4) *Xanthomonas holcicola*, including *X. campestris* pv. *holcicola* and *X. campestris* pv. *vasculorum* B;

(5) *Xanthomonas bromi*, for the *Xanthomonas* isolates from *Bromus*;

(6) *Xanthomonas sacchari*, for the strains isolated from sugarcane in Guadeloupe, and possibly including the single Indian strain from sugarcane.

Some strains designated as *X. axonopodis* (i.e. type B) are misclassified as *Xanthomonas*.

As the DNA hybridization work within the genus *Xanthomonas* is still ongoing, it is possible that other *X. campestris* pathovars will be shown to share high DNA homology with one of the aforementioned groups. According to the rules of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975), the earliest valid specific epithet should be used for naming a species. It may thus be premature to propose formal reclassifications at this stage, as this could result in subsequent nomenclatural changes. The new species suggested here should therefore be considered as provisional entities.

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