Differentiation between *Xanthomonas campestris* pv. *oryzae*, *Xanthomonas campestris* pv. *oryzicola* and the Bacterial 'Brown Blotch' Pathogen on Rice by Numerical Analysis of Phenotypic Features and Protein Gel Electrophoregrams

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Thirty-five *Xanthomonas campestris* pv. *oryzae*, fourteen *X. campestris* pv. *oryzicola* strains and six 'brown blotch' pathogens of rice, all of different geographical origin, were studied by numerical analysis of 133 phenotypic features and gel electrophoregrams of soluble proteins, %G + C determinations and DNA:rRNA hybridizations. The following conclusions were drawn. (i) The *Xanthomonas campestris* pathovars *oryzae* and *oryzicola* display clearly distinct protein patterns on polyacrylamide gels and can be differentiated from each other by four phenotypic tests. (ii) Both pathovars are indeed members of *Xanthomonas* which belongs to a separate rRNA branch of the second rRNA superfamily together with the rRNA branches of *Pseudomonas fluorescens*, *Marinomonas*, *Azotobacter*, *Azomonas* and *Frateriuria*. (iii) 'Brown blotch' strains are considerably different from *X. campestris* pv. *oryzae* and *oryzicola*. They are not members of the genus *Xanthomonas*, but are more related to the generically misnamed *Flavobacterium capsulatum*, *Pseudomonas paucimobilis*, *Flavobacterium devorans* and '*Pseudomonas azotocolligans*' belonging in the fourth rRNA superfamily. (iv) No correlation was found between the virulence, pathogenic groups or geographical distribution of *X. campestris* pv. *oryzae* or pv. *oryzicola* strains and any phenotypic or protein electrophoretic property or clustering.

INTRODUCTION

Bacterial blight of rice, caused by *Xanthomonas campestris* pv. *oryzae* (Ishiyama 1922) Dye 1978, is a major problem for the cultivation of rice in Asia (Ou, 1972). The disease has also been reported in Northern Australia (Aldrick *et al.*, 1973), Latin America (Lozano, 1977) and Africa (Buddenhagen *et al.*, 1979; Reckhaus, 1983). Present knowledge on the symptomatology, epidemiology and control of the disease, as well as the morphology, cytology, physiology, toxin production, host range, serology, pathogenic variability and phage sensitivity of its causal agent have been reviewed (Mizukami & Wakimoto, 1969; Anonymous, 1970; Ou, 1972). Several authors have tried to classify *X. campestris* pv. *oryzae* strains on the basis of their phage sensitivity (Wakimoto, 1960), serological properties (Choi *et al.*, 1980), phenotypic features (Shekhawat & Srivastava, 1968; Hifni *et al.*, 1975; Reddy & Ou, 1976; Tsuchiya *et al.*, 1982*a*) and pathogenic variability (Ezuka & Sakaguchi, 1978; Mew & Vera Cruz, 1979). Serovar specificity of the strains is highly correlated with their phage sensitivity (Tsuchiya *et al.*, 1982*b*). However, no correlation was found between the pathogenic variability and any of the phenotypic features of the strains (Hifni *et al.*, 1975; Tsuchiya *et al.*, 1982*a*).

Leaf streak, caused by *X. campestris* pv. *oryzicola* (Fang *et al.*, 1957) Dye 1978, is another economically important rice disease widespread in tropical Asia. It has not been reported from Japan or other temperate regions (Ou, 1972).

Lozano *et al.* (1981) reported the occurrence of a new bacterial rice disease in Central and Latin America, designated as 'brown blotch'. The pathogen was assigned to the genus *Xanthomonas* but a subgeneric identification was not provided.

The disease patterns caused by the three pathogens are well defined and can be differentiated easily from each other. In the seedbed, *X. campestris* pv. *oryzae* causes tiny water-soaked spots at the margins of fully developed leaves. These spots enlarge and turn yellow, and the plant finally dries and wilts. In the case of adult plants, lesions are first observed at the margin near the tip as water-soaked strips which have a waxy margin, enlarge, turn yellow and finally cover the entire leaf, which becomes greyish due to saprophytic growth (Ou, 1972). Bacterial leaf streak is characterized initially by fine translucent streaks along the leaves, over the larger veins. Later, the lesions turn brown, spreading over the entire leaf, which dies (Ou, 1972). The symptoms of 'brown blotch' disease comprise the spreading and enlargement of brown blotches on the leaves (Lozano *et al.*, 1981).

Bacterial rice pathogens have been studied mostly from a phytopathological point of view. Bacteriological studies on *X. campestris* pv. *oryzae* and pv. *oryzicola* have been published by Mukoo & Isaka (1964), Goto (1964), Watanabe (1966), Shekhawat & Srivastava (1968), Reddy & Ou (1974, 1976), Hifni *et al.* (1975) and Tsuchiya *et al.* (1982a). An extensive comparative taxonomic study has not been carried out up to now.

The objectives of the present study were: (1) to determine the degree of phenotypic similarity of 35 *X. campestris* pv. *oryzae*, 14 *X. campestris* pv. *oryzicola* strains and 6 'brown blotch' isolates of different geographical origin by numerical analysis of 133 phenotypic features; (2) to determine the fine internal structure of these taxa by numerical analysis of polyacrylamide gel electrophoregrams; (3) to determine the generic affiliation of the unnamed 'brown blotch' isolates by DNA:rRNA hybridizations; (4) to construct a diagnostic key for the differentiation of *X. campestris* pv. *oryzae*, *X. campestris* pv. *oryzicola* and the 'brown blotch' isolates from each other; (5) to verify whether there is any correlation between the virulence of the strains and some of their phenotypic or protein electrophoretic properties.

METHODS

Organisms used. The strains (Table 1) were grown at 28 °C on GYCA medium, consisting of 1% (w/v) D-glucose, 0.5% (w/v) yeast extract (Oxoid), 3% (w/v) CaCO₃ and 2.5% (w/v) agar in tap water (modified from Dye, 1962). They were maintained at 4 °C on the same medium. The cultures used for the phytopathological tests on rice variety TN1 were grown on Wakimoto's potato semi-synthetic medium containing 300 g potato, 0.5 g Ca(NO₃)₂·4H₂O, 2 g Na₂HPO₄·12H₂O, 5 g peptone, 20 g sucrose and 15 g agar in 1 litre of distilled water (Ou, 1972).

Morphological, physiological and biochemical features. Gram-staining was performed on cells grown for 48 h on GYCA medium. Two basal media were used: (1) YS medium containing 0.05% (w/v) NH₄H₂PO₄, 0.05% (w/v) K₂HPO₄, 0.02% (w/v) MgSO₄·7H₂O, 0.5% (w/v) NaCl and 0.5% yeast extract (Oxoid) in distilled water (Dye, 1962) and (2) GYEA medium containing 0.5% D-glucose, 0.1% yeast extract (Oxoid), a trace of FePO₄ and 2% agar (Dickinson *et al.*, 1975). Indole production was detected after 5 d by Kovacs' (1928) reagent in a medium containing 0.5% yeast extract and 2% (w/v) tryptone (Oxoid) (Dye, 1962; modified). Acetoin formation was observed in YS medium supplemented with 0.5% D-glucose, after 5 d, using Barritt's (1936) reagent. Urease activity was tested according to Dye (1962). Gelatinase activity was detected in GYEA medium in tubes with 2% agar replaced by 12% (w/v) gelatine. The tubes were stab-inoculated and the results were observed after 14 d. Reactions in litmus milk, i.e. strong peptonization, alkalization and reduction, were observed after 14 d incubation. Starch hydrolysis was detected by streaking a loopful of cells on a medium containing 0.5% (w/v) neutralized peptone (Oxoid), 0.3% yeast extract, 0.2% (w/v) soluble starch and 2% agar in distilled water. Lugol's iodine solution was poured on the plates after 7 d incubation. For the aesculin and arbutin hydrolysis tests, a medium consisting of GYEA supplemented with 0.05% (w/v) ferric ammonium citrate and either 0.3% (w/v) aesculin or 0.3% (w/v) arbutin, respectively, was inoculated by streaking a loopful of cells on the medium. A black zone (in visible light) and a clear zone (in UV light) around the growth streak indicated a positive reaction. Pectinase activity was tested using Beraha's (1968) procedure after 14 d incubation. The production of 2-ketogluconic acid by resting cells in a 2% (w/v) sodium gluconate solution in 0.01 M-phosphate buffer, pH 7.0, was detected after 7 d incubation in a shaking apparatus by thin-layer chromatography using *o*-phenylenediamine hydrochloride as spray reagent. This reagent yields a blue fluorescent colour with 2-ketogluconic acid upon observation of the chromatograms under UV light 16 h after spraying (Gosselé *et al.*, 1980). Phenylalanine deaminase activity was detected on slants con-

sisting of 0.3% yeast extract, 0.1% (w/v) Na_2HPO_4 , 0.5% NaCl and 2% agar; L-phenylalanine was added separately to a final concentration of 0.1% (w/v). After 7 d incubation, 10% (w/v) FeCl_3 solution was added and the development of a green colour indicated the presence of phenylpyruvate (Ewing *et al.*, 1957). We used the method of Dye (1962) for testing the tyrosinase activity after 14 d incubation in a shaking apparatus. The production of H_2S from L-cysteine or peptone was detected with lead(II) acetate paper strips in YS medium (NaCl omitted) containing 0.01% (w/v) cysteine or 0.02% peptone, respectively. Hydrolysis of Tween 20, 40, 60 and 80 was observed after 4 d incubation on a medium consisting of 1% peptone (Difco), 0.01% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1% yeast extract and 2% agar with the Tweens added separately to a final concentration of 1% (v/v). Egg yolk hydrolysis was checked on GYEA medium supplemented with 1% NaCl and 10% (v/v) egg yolk solution (Oxoid). Kovacs' (1956) reagent was used for the oxidase test. Reduction of 0.001% (w/v) K_2TeO_3 in GYEA was indicated by a blackening of the medium. The oxidation-fermentation (O/F) test was performed by stab-inoculation in a medium containing 0.2% peptone, 0.5% NaCl, 0.03% K_2HPO_4 , 0.003% (w/v) bromocresolpurple, 1% D-glucose and 0.3% agar in distilled water. Nitrate reduction was detected in Dye's (1962) medium from which NaCl was omitted. Acid formation from carbohydrates (1%, w/v) was followed in YS-medium (NaCl omitted), with 0.001% bromocresol purple as indicator. Growth on carbohydrates (0.2%, w/v), alcohols (0.2%, w/v), polyphenolic compounds (0.1%, w/v), glycosides (0.1%, w/v), organic acids (0.1%, w/v), DNA bases (0.1%, w/v) and L-alanine (0.1%, w/v) was tested in Starr's (1945) modified medium. The basal medium consisted of 0.005% yeast extract, 0.1% NH_4Cl , 0.2% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2% agar, and was adjusted to pH 7.0. A solution of trace elements, adjusted to pH 7.0 and filter-sterilized, was added separately in order to obtain the following quantities per 100 ml of medium: 2.5 μg H_3BO_3 , 50 μg CaCO_3 , 5 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.5 μg KI, 5 μg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 5 μg MoO_3 and 25 μg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The carbon sources to be tested were filter-sterilized; stock solutions of all carbon sources, with the exception of carbohydrates, were adjusted to pH 7.0 before sterilization. Temperature tolerance was observed in a medium containing 0.5% D-glucose, 0.5% yeast extract and 0.5% neutralized peptone in distilled water. Growth in anaerobic conditions was tested on GYEA medium using an anaerobic jar with the Gas-pak system (Oxoid). We followed Dye's (1962) procedure for testing growth on L-asparagine as sole source of carbon and nitrogen. Growth tests in the presence of antibiotics, salts and dyes were done on GYEA medium and evaluated after 7 d incubation. Growth on 0.2% (w/v) vitamin-free Casamino acids (supplemented with 2.5% agar) was evaluated after 7 d incubation.

The suspensions used for the inoculation of liquid media were prepared in distilled water from cells grown for 48 h on GYCA medium and contained about 10^8 cells per ml. Two droplets were added to the liquid media, which were incubated on a shaking apparatus for 7 d, unless stated otherwise. Solid media in Petri dishes were inoculated with a multipoint inoculator and incubated for 7 d. The incubation temperature was always 28 °C, except for gelatinase (20 °C) and temperature-tolerance tests (4, 10, 32 and 35 °C).

Phytopathological tests. The pathogenicity of all *Xanthomonas campestris* pv. *oryzae* and pv. *oryzicola* strains was tested on the susceptible rice cultivar TN1. Seedlings were grown in wooden seedboxes and transplanted 10 d after sowing into clay pots at three seedlings per pot. The inoculum was prepared by suspending a 3-d-old bacterial culture, grown on Wakimoto's medium (see above), in 10 ml sterile distilled water at approximately 10^9 cells ml⁻¹. Inoculations were performed 40 d after sowing. Two inoculation methods were applied: (1) the three youngest unfolded leaves from each tiller were inoculated by cutting 1 to 2 cm from the tip with a pair of scissors, dipped in the inoculum (leaf-clipping method of Kauffman *et al.*, 1973); (2) the three youngest leaves of another plant were inoculated by rubbing the surface of the blades from the bottom to the tip with a gauze dipped in the inoculum (leaf-rubbing method). The results were scored after 14 d by determining the percentage leaf area infected by bacterial blight and bacterial leaf streak symptoms based on the Standard Evaluation System for Rice (Anonymous, 1980).

Unpeeled potatoes were washed thoroughly with tap water and surface-sterilized using the following procedure: immersion in 70% (v/v) alcohol for 1 min, flame burning, immersion in fivefold diluted bleach for 5 min, washing in 0.1 M-HCl and two washings in sterile distilled water. The surface-sterilized potatoes were cut with a sterile knife into slices, which were put into Petri dishes on sterile filter paper (Whatman no. 1), moistened with sterile water. Carrots were immersed for 1 min in 70% alcohol and then flamed. Surface-sterilized carrot and potato slices were infected with one droplet of the bacterial suspension and inspected for soft-rot after 2 d incubation at 28 °C. Sterile water and a soft-rot-causing *Erwinia carotovora* strain were included as controls. All tests were done in duplicate.

Numerical analysis of phenotypic features. All 133 phenotypic features were coded as 2 (positive) or 1 (negative). Missing features (an average of 0.6 tests per strain) were encoded as 0 (no comparison). For the reaction in litmus milk, three states were distinguished: strong peptonization, alkalization and reduction. Similarities between the strains were calculated with the similarity coefficient of Sokal & Michener (1958) (S_{SM}) and Jaccard's coefficient (S_J) using a modified program of Bonham-Carter (1967). The clustering of the strains was performed according to the unweighted average pair-group method (Sneath & Sokal, 1973) with the Clustan 1C program (Wishart, 1978) using the Siemens 7541 (BS2000) computer of the Centraal Digitaal Rekencentrum, RUG, Gent, Belgium.

Numerical analysis of protein gel electrophoregrams. Strains were grown in Roux flasks on GYCA medium at

Table 1. Designation, source and phytopathological reactions of strains assigned to phenons and clusters in the phenotypic and protein electrophoretic analysis

Phenon and electro-phoretic cluster	Pathovar names are according to Dye <i>et al.</i> (1980)					Mean percentage of leaf area infected by bacterial blight and organisms on rice variety TN14
	Strain no.	Name	Other strain designation(s)	Isolation place and year	Source†	
1	NCPB 1152	<i>Xanthomonas campestris</i> pv. <i>oryzae</i>	PDDCC 443	Japan	ex N. Okabe	5
	NCPB 1153	<i>X. campestris</i> pv. <i>oryzae</i>	PDDCC 442	Japan	ex H. Mukoo	4
	NCPB 1154	<i>X. campestris</i> pv. <i>oryzae</i>	PDDCC 440	Japan	ex H. Mukoo	4
	NCPB 1936	<i>X. campestris</i> pv. <i>oryzae</i>	ICPB X0110	India, 1963	ex D. N. Srivastava	42
	NCPB 2446	<i>X. campestris</i> pv. <i>oryzae</i>	E698	Japan, 1958	S. Yoshimura	8
	T7174	<i>X. campestris</i> pv. <i>oryzae</i>		Ooita, Japan	S. Wakimoto	7
	T7133	<i>X. campestris</i> pv. <i>oryzae</i>		Mie, Japan	S. Wakimoto	58
	Q7472	<i>X. campestris</i> pv. <i>oryzae</i>		Ooita, Japan	S. Wakimoto	31
	Q7527	<i>X. campestris</i> pv. <i>oryzae</i>		Miyazaki, Japan	S. Wakimoto	8
	H75304	<i>X. campestris</i> pv. <i>oryzae</i>		Miyazaki, Japan	S. Wakimoto	12
	T7147	<i>X. campestris</i> pv. <i>oryzae</i>		Japan	S. Wakimoto	1
	IRN 198	<i>X. campestris</i> pv. <i>oryzae</i>		Bolivia	H. Kauffmann	10
	IRN 279	<i>X. campestris</i> pv. <i>oryzae</i>		Nepal	ex T. W. Mew	45
	IRN 325	<i>X. campestris</i> pv. <i>oryzae</i>		Indonesia	ex T. W. Mew	60
	PDDCC 3123	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1963	ex Y. P. Rao	8
	PDDCC 3124	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1965	ex Y. P. Rao	8
	PDDCC 3125*	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1965	ex Y. P. Rao	NT
	PDDCC 3126	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1966	ex Y. P. Rao	8
	PDDCC 3127	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1966	ex Y. P. Rao	4
	PDDCC 3128	<i>X. campestris</i> pv. <i>oryzae</i>		India, 1970	ex Y. P. Rao	34
	PDDCC 4646	<i>X. campestris</i> pv. <i>oryzae</i>		China	ex T. C. Huang	32
	PXO 10	<i>X. campestris</i> pv. <i>oryzae</i>		Philippines	M. P. Natural	50
	PXO 40	<i>X. campestris</i> pv. <i>oryzae</i>		Philippines	ex T. W. Mew	11
	PXO 61	<i>X. campestris</i> pv. <i>oryzae</i>		Philippines	ex T. W. Mew	42
	PXO 71	<i>X. campestris</i> pv. <i>oryzae</i>		Philippines, 1973	T. W. Mew	9
	PXO 79	<i>X. campestris</i> pv. <i>oryzae</i>		Palawan area, Philippines, 1974	ex T. W. Mew	10
	PXO 82	<i>X. campestris</i> pv. <i>oryzae</i>		Davao, Philippines, 1963	ex T. W. Mew	39
	PXO 86	<i>X. campestris</i> pv. <i>oryzae</i>		Philippines	ex T. W. Mew	37
	CIAT 1185	<i>X. campestris</i> pv. <i>oryzae</i>		Colombia	J. C. Lozano	33
	CIAT 1186	<i>X. campestris</i> pv. <i>oryzae</i>		Colombia	J. C. Lozano	39
	E698	<i>X. campestris</i> pv. <i>oryzae</i>	T7174	Japan	S. Wakimoto	8
	21	<i>X. campestris</i> pv. <i>oryzae</i>		West Java, Indonesia	T. Suparyono	20
	37	<i>X. campestris</i> pv. <i>oryzae</i>		Central Java, Indonesia	T. Suparyono	25
	59	<i>X. campestris</i> pv. <i>oryzae</i>		West Java, Indonesia	T. Suparyono	35

Table 1. (continued)

2§	IRN 235§	<i>X. campestris</i> pv. <i>oryzae</i>		Kogoni, Mali		32
2	NCPPB 11501	<i>X. campestris</i> pv. <i>oryzicola</i>	PDDCC 364	Philippines	ex F. T. Orillo	5
	NCPPB 11511	<i>X. campestris</i> pv. <i>oryzicola</i>	PDDCC 363	Philippines	ex F. T. Orillo	<1
	NCPPB 1585*	<i>X. campestris</i> pv. <i>oryzicola</i>	PDDCC 5743	Malaysia, 1964	A. C. Hayward	25
			ICPB X0111			
	NCPPB 1632	<i>X. campestris</i> pv. <i>oryzicola</i>	ICPB X0109	Philippines, 1963	ex M. Goto	0
	PDDCC 1621	<i>X. campestris</i> pv. <i>oryzicola</i>	ICPB X0104	Philippines, 1963	ex M. Goto	25
	PDDCC 1622	<i>X. campestris</i> pv. <i>oryzicola</i>	ICPB X0105	Philippines, 1963	ex M. Goto	0
	PDDCC 3129	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1967	ex G. S. Shekhawat	0
	PDDCC 3130	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1967	ex G. S. Shekhawat	0
	PDDCC 3131	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1967	ex G. S. Shekhawat	<1
	PDDCC 3133	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1967	ex G. S. Shekhawat	<1
	PDDCC 3134	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1966	ex G. S. Shekhawat	5
	PDDCC 3135	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1968	ex G. S. Shekhawat	<1
	PDDCC 3136	<i>X. campestris</i> pv. <i>oryzicola</i>		India, 1968	ex G. S. Shekhawat	0
	PDDCC 4654	<i>X. campestris</i> pv. <i>oryzicola</i>	CCM 2760	Philippines, 1976	ex T. W. Mew	25
3	CIAT 1171	'Brown blotch' isolate		Norte, Panama, 1977	J. C. Lozano	NT
	CIAT 1173	'Brown blotch' isolate		Monteria-Cordoba, Colombia, 1977	J. C. Lozano	NT
	CIAT 1187	'Brown blotch' isolate		Natama-Tolima, Colombia, 1978	J. C. Lozano	NT
	CIAT 1191					
	tl, 12†	'Brown blotch' isolate		Monteria-Cordoba, Colombia, 1978	J. C. Lozano	NT
	CIAT 1192	'Brown blotch' isolate		Natama-Tolima, Colombia, 1978	J. C. Lozano	NT

NT, Not tested.

Sources: CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; ICPB, International Collection of Phytopathogenic Bacteria, Davis, California, USA; IRN, Isolation Record Number at IRRI, Los Baños, the Philippines; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; PDDCC, Plant Diseases Division Culture Collection, Auckland, New Zealand. Strain PDDCC 3125 was not investigated in the phenotypic analysis.

* Neopathotype strains according to Dye *et al.* (1980).

† Names of isolators are in italics; names preceded by 'ex' indicate individuals who successively maintained the culture before its deposition.

‡ Determined according to the Standard Evaluation System for Rice (Anonymous, 1980).

§ Strain IRN 235 belongs phenotypically in phenon 2; it causes bacterial blight symptoms and occupies a separate position according to its protein electrophoregrams (see Figs 1 and 2).

|| Strains NCPPB 1150 and NCPPB 1151 have been named *X. campestris* pv. *oryzae*. However, pathogenicity tests clearly showed that both strains are leaf streak organisms. Their PDDCC equivalents, i.e. PDDCC 364 and PDDCC 363, respectively, have not been examined.

¶ Strains CIAT 1191, showing two colony types (tl, small; t2, large) was isolated from *Echinocloa colonum*.

28 °C for 40 h and cells were harvested with 0.01 M-phosphate buffer, pH 7.0, vigorously stirred in a mixer (Ultra-Turrax, Janke & Kunkel, Staufen, FRG) in order to remove the polysaccharides, centrifuged at 10000 *g* for 10 min, and washed twice with 3 mM-Tris/HCl buffer, pH 7.0. Cell-free extracts were prepared as described previously (Kerstens & De Ley, 1975), and the soluble protein fraction was dialysed against 1 litre of 6.4 mM-Tris/HCl buffer, pH 9.2. Polyacrylamide gel electrophoresis, densitometry, photography of the stained gels and normalization of the densitometric tracings were performed by the method of Kersters & De Ley (1975). Each protein extract was investigated in at least two different electrophoretic experiments. Each normalized densitometric tracing was converted into a sequence of 110 numbers, representing the optical density (expressed as height in mm) of each protein on the scan. Computer-assisted calculations of the Pearson product-moment correlation coefficient (*r*) and clustering by the unweighted average pair-group method were performed as described previously (Kerstens & De Ley, 1975) using the Clustan 1C program (Wishart, 1978).

DNA base composition. The average mol%G + C content of the overall genome DNA of *X. campestris* pv. *oryzae* NCPPB 1153 and T7147, *X. campestris* pv. *oryzicola* NCPPB 1585 and the 'brown blotch' isolates CIAT 1171 and CIAT 1173 was measured by the thermal denaturation method (De Ley & Van Mylem, 1963) and calculated by the equation of Marmur & Doty (1962), as modified by De Ley (1970).

DNA:rRNA hybridizations. The preparation of DNA, fixation of single-stranded high molecular weight DNA on membrane filters, chemical determination of the amount of DNA on the filters, the preparation of [¹⁴C]rRNA, and the hybridizations between filter-fixed DNA and 16S or 23S [¹⁴C]rRNA were carried out as described by De Vos & De Ley (1983). Reference [¹⁴C]rRNAs were prepared from type strains of *X. campestris* pv. *campestris* (NCPPB 528) and *Flavobacterium capsulatum* (ATCC 14666). From each DNA:rRNA hybrid two parameters were measured: (1) *T*_{m(e)}, which is the temperature at which 50% of the hybrid is denatured; (2) the percentage of rRNA binding, representing the amount of labelled rRNA duplexed, in µg per 100 µg DNA fixed on the filter, after ribonuclease treatment.

RESULTS

Numerical analysis of phenotypic features

The dendrogram obtained after numerical analysis of 133 unit characters of 34 *X. campestris* pv. *oryzae*, 14 *X. campestris* pv. *oryzicola* and 6 'brown blotch' strains, using the *S*_{SM} coefficient and the unweighted average pair-group method, is shown in Fig. 1(a). At the 73% *S*_{SM} level, three phenons can be differentiated. Thirty-three *X. campestris* pv. *oryzae* strains cluster together at 78% *S*_{SM} in phenon 1. Phenon 2 contains fourteen *X. campestris* pv. *oryzicola* strains uniting at the 85% *S*_{SM} level and one *X. campestris* pv. *oryzae* strain (IRN 235). The latter strain is only loosely linked to phenon 2 at 74% *S*_{SM} (Fig. 1a). Phenon 3 contains all six 'brown blotch' isolates, clustering together at the 90% *S*_{SM} level. The same three phenons were recovered when the *S*_J coefficient was used.

Morphological, physiological and biochemical features

The following 19 features were uniformly present or gave a positive reaction in all 54 strains examined: cells occurring singly; starch hydrolysis; catalase; oxidative metabolism; acid from D-glucose; growth on D-glucose, D-galactose, cellobiose, sucrose, sodium fumarate, sodium L-malate, sodium succinate; weak growth at 32 °C and 10 °C (not tested for the 'brown blotch' isolates); growth in the presence of 0.001% (w/v) trimethoprim, 0.005% (w/v) metronidazole, 0.001% (w/v) auramin O, 0.0025% (w/v) thionine, 0.1% (w/v) actidione. The latter two features have not been tested for the 'brown blotch' isolates.

The following 55 features were uniformly absent or gave a negative reaction in all 54 strains: Gram-reaction; indole production; urease production; 2-ketogluconic acid formation; hydrolysis of egg yolk; nitrate reduction; acid from D-ribose, L-rhamnose, salicin, sorbitol, *meso*-inositol, dulcitol, adonitol, maltose, lactose, inulin; growth on L-arabinose, D-ribose, L-rhamnose, lactose, raffinose, inulin, methanol, ethanol, *n*-propanol, ethanediol, *meso*-inositol, sorbitol, dulcitol, adenine, guanine, cytosine, thymine, sodium potassium tartrate, sodium

Fig. 1. Dendrograms showing the relationships between *Xanthomonas campestris* strains and 'brown blotch' strains isolated from rice. (a) Phenogram based on the *S*_{SM} coefficient and the unweighted average linkage algorithm. (b) Clustering of the protein electrophoregrams of the strains based on the correlation coefficient *r* and the unweighted average linkage algorithm. Neopathotype strains proposed by Dye *et al.* (1980) are marked *.

Table 2. *Differential phenotypic features between X. campestris pv. oryzae (phenon 1), X. campestris pv. oryzicola (phenon 2) and 'brown blotch' isolates (phenon 3)*

Phenon number No. of strains	Percentage of strains positive		
	1 33	2 15	3 5
Cells occurring in:			
pairs	100*	100*	0
chains	67	67	0
Occurrence of filaments	55	60	17
Oxidase	0	0	100
Acetoin production	0	93	0
Gelatinase activity	49	100	0
Reactions in litmus milk:			
strong peptonization	0	87	0
alkalinization	97	100	0
reduction	94	100	0
Aesculin hydrolysis	100	100	33
Phenylalanine deaminase	0	53	0
Tyrosinase	0	7	0
H ₂ S formation from:			
peptone	100	100	0
L-cysteine	100	100	67
Hydrolysis of:			
Tween 20	88	27	17
Tween 40	97	100	100
Tween 60 or Tween 80	100	100	0
Arbutin hydrolysis	91	67	0
Pectinolysis	6	27	0
Reduction of potassium tellurite	39	7	100
Acid formation from:			
L-arabinose	27	53	0
cellobiose	100	100	0
D-fructose	91	93	100
D-galactose	97	100	0
D-mannose	85	93	67
sucrose	79	100	0
trehalose	94	94	0

glyoxylate, 2-ketogluconic acid, tannic acid, *p*-hydroxybenzoic acid, hydroquinone, phloroglucinol, resorcinol, amygdalin, arbutin, aesculin; growth at 4 °C or 35 °C; growth in anaerobic conditions; growth in the presence of 0.001% (w/v) tetracycline hydrochloride, 0.005% (w/v) chloramphenicol, 0.001% (w/v) novobiocin, 0.001% (w/v) doxycycline, 3% NaCl, 0.001% (w/v) CGA 78039 (7-chloro-6-fluoro-1,4-dihydro-4-oxo-1-ethyl-3-quinoline carboxylic acid), 0.05% (w/v) crystal violet; growth on L-asparagine as sole source of carbon and nitrogen.

The results of the tests giving differential reactions are given in Table 2. Some tests such as acid formation from and growth on carbon sources, hydrolysis of Tweens, acetoin formation and growth in the presence of inhibitors were repeated for all strains. The average reproducibility was 96%.

Phytopathological properties

The phytopathogenicity of the strains towards rice variety TN1 is indicated in Table 1. No strain showed any reaction on carrot or potato slices.

Numerical comparison of protein gel electrophoregrams

More than 150 normalized protein patterns of the 55 strains investigated (Table 1) were compared by numerical analysis. The reproducibility limits were above $r = 0.95$ for 48 strains and above $r = 0.93$ for the remaining 7 strains. The most typical electrophoregram of each strain (Swings *et al.*, 1976) was used for the final clustering shown in Fig. 1(b). At $r = 0.89$, three

Table 2.(continued)

Phenon number No of strains	Percentage of strains positive		
	1 33	2 15	3 5
Growth on sole carbon source:			
L-alanine	0	93	100
D-fructose	94	94	100
glycerol	0	0	100
sodium aconitate	12	60	0
sodium L-lactate	100	100	0
sodium oxaloacetate	100	100	83
sodium propionate	0	0	100
trehalose	94	100	100
trisodium citrate	73	100	0
D-xylose	100	100	0
Growth in the presence of (% w/v):			
amoxicillin (0.001)	3	60	0
ampicillin (0.001)	3	93	17
cadmium acetate (0.0001)	9	67	33
cupric nitrate (0.001)	97	7	100
erythromycin (0.001)	0	7	100
8-hydroxyquinoline (0.001)	0	0	100
methicillin (0.005)	0	0	100
p-methylaminophenol sulphate (0.005)	70	100	100
neomycin (0.005)	15	93	67
potassium tellurite (0.001)	49	13	100
rifampicin (0.001)	0	7	67
sodium chloride (0.5-2)	97	100	100
sodium formaldehyde sulfoxylate (0.005)	46	33	100
streptomycin (0.001)	30	100	100
2,4,6-trichlorophenol (0.005)	15	100	100
zinc oxide (0.001)	0	0	100
Growth on 0.2% vitamin-free Casamino acids after 7 d	0	100	100

* Although the majority of the cells occurred singly, cells in pairs were also observed in each strain.

electrophoretic clusters could be delineated, corresponding to *X. campestris* pv. *oryzae*, pv. *oryzicola* and the 'brown blotch' isolates. One strain of *X. campestris* pv. *oryzae* (IRN 235) occupied a separate position in the dendrogram. According to the *r*-matrix (not shown) its closest gel electrophoretic neighbours were *X. campestris* pv. *oryzae* strains T7133, Q7472, H75304 and PDDCC 3128.

The electrophoregrams of strains belonging to each cluster were always very similar upon visual inspection (Fig. 2). The neopathotype strain PDDCC 3125 (= NCPPB 3002) of *X. campestris* pv. *oryzae* was not included in the phenotypic analysis, but appeared to be a normal member of *X. campestris* pv. *oryzae* according to its electrophoretic protein pattern (Fig. 1b, Fig. 2).

DNA base composition and DNA:rRNA hybridizations

The mol% G + C values of the DNAs of two strains of *X. campestris* pv. *oryzae*, one strain of *X. campestris* pv. *oryzicola* and two 'brown blotch' isolates are given in Table 3. The results of the hybridizations of DNA of these strains versus [¹⁴C]rRNA of the type strains of *X. campestris* pv. *campestris* (NCPPB 528) and *Flavobacterium capsulatum* (ATCC 14666) are compiled in Table 3, together with the $T_{m(e)}$ values and percentages of rRNA binding of the homologous duplexes of these type strains. Hybrids of reference ¹⁴C-labelled 23S rRNA from *X. campestris* pv. *campestris* NCPPB 528 with DNAs of two strains of *X. campestris* pv. *oryzae* and one strain of pv. *oryzicola* had a thermal denaturation midpoint temperature ($T_{m(e)}$) range of 80.2 to 80.4 °C (Table 3). These $T_{m(e)}$ values prove that the generic assignment of *X. campestris* pv. *oryzae* and

Fig. 2. Normalized protein electrophoregrams from 14 *Xanthomonas campestris* strains and 2 'brown blotch' strains isolated from rice. Lanes (a)–(h): *X. campestris* pv. *oryzae* IRN 235, PDDCC 3125 [neopathotype strain proposed by Dye *et al.* (1980)], PXO 61, PXO 71, PXO 79, PXO 86, NCPPB 1153, IRN 325. Lanes (i)–(n): *X. campestris* pv. *oryzicola* NCPPB 1150, NCPPB 1585 [neopathotype strain proposed by Dye *et al.* (1980)], PDDCC 1622, PDDCC 3130, PDDCC 3131, PDDCC 3135. Lanes (o) and (p): 'brown blotch' isolates CIAT 1187 and CIAT 1192.

Table 3. DNA base composition of some *X. campestris* pv. *oryzae*, pv. *oryzicola* and 'brown blotch' isolates; results of hybridizations between DNA of these strains and ^{14}C -labelled 23S rRNA from the type strains of *X. campestris* pv. *campestris* and 16S rRNA of *Flavobacterium capsulatum*

Organism used for DNA isolation	Origin and strain no.	Mol% G + C	Hybridization with [^{14}C]rRNA of:			
			<i>X. campestris</i> NCPPB 528		<i>F. capsulatum</i> ATCC 14666	
			$T_{m(e)}$ (°C)	% rRNA binding	$T_{m(e)}$ (°C)	% rRNA binding
<i>X. campestris</i> pv. <i>campestris</i>	NCPPB 528*	65.2†	81.0†	0.09†		
<i>X. campestris</i> pv. <i>oryzae</i>	NCPPB 1153	64.5	80.3	0.06		
<i>X. campestris</i> pv. <i>oryzae</i>	T7147	64.7	80.4	0.07		
<i>X. campestris</i> pv. <i>oryzicola</i>	NCPPB 1585*	65.0	80.2	0.07		
'Brown blotch' isolate	CIAT 1171	63.2	58.4	0.08	76.2	0.10
'Brown blotch' isolate	CIAT 1173	63.5	56.1	0.06	75.6	0.09
<i>F. capsulatum</i>	ATCC 14666*	63‡			81.0§	0.17§

* Type or neopathotype strains.

† From De Vos & De Ley (1983).

‡ From Bradbury (1984).

§ From Bauwens (1980).

pv. *oryzicola* is correct. However, in the same conditions, both 'brown blotch' isolates had $T_{m(e)}$ values of only 56.1 °C and 58.4 °C, indicating that these strains do not belong to the genus *Xanthomonas*, which is a member of the second rRNA superfamily (*sensu* De Ley, 1978). The relatively high $T_{m(e)}$ values of 75.6 °C and 76.2 °C of the hybrids of DNA from the 'brown blotch' isolates

with reference ^{14}C -labelled 16S rRNA from the type strain of *Flavobacterium capsulatum* (ATCC 14666) show that these strains belong to the rRNA branch of *F. capsulatum*, which is a member of rRNA superfamily IV (see below).

DISCUSSION

Morphological, physiological and biochemical features

All *X. campestris* pv. *oryzae* and pv. *oryzicola* strains grew luxuriantly on GYCA medium showing typical yellow-pigmented colonies. On this medium 'brown blotch' isolates produced a somewhat darker yellow non-diffusible pigment.

According to Dye (1962), acetoin is not produced by *Xanthomonas*. However, in the case of *X. campestris* pv. *oryzicola*, which was not studied by Dye (1962), a moderate reaction was obvious. In order to test the gelatinase activity we used the classical stab-inoculation method, since Oxoid charcoal discs inhibited the growth of most *X. campestris* pv. *oryzae* strains. All phenon 2 strains showed a strong gelatinase activity, whereas in phenon 1, some strains were weakly positive or even negative. 'Brown blotch' isolates showed no gelatinase activity. There is no correlation between the gelatinase activity of *X. campestris* pv. *oryzae* isolates and their pathogenicity towards rice variety TN1, confirming the conclusions of Shekhawat & Srivastava (1968), Hifni *et al.* (1975) and Tsuchiya *et al.* (1982a), but contradicting these from Mukoo & Isaka (1964).

X. campestris pv. *oryzicola* effects a strong peptonization of litmus milk, together with an alkalization starting at the top of the medium and a reduction starting from the bottom. In the case of *X. campestris* pv. *oryzae*, the peptonization was weak, but a clear alkalization and reduction were observed. 'Brown blotch' isolates showed only a weak peptonization. An alkaline reaction in litmus milk caused by the blight pathogen was also reported by Goto (1964), Mukoo & Isaka (1964), Reddy & Ou (1976) and Tsuchiya *et al.* (1982a).

All strains hydrolysed starch weakly after 7 d. This is in contradiction with the results of Hifni *et al.* (1975) and Reddy & Ou (1976), who observed no amylase activity. All *X. campestris* pv. *oryzae* and pv. *oryzicola* strains hydrolysed aesculin, whereas only two 'brown blotch' strains (CIAT 1191t1 and t2) gave a positive reaction. Lozano *et al.* (1981) did not detect aesculin hydrolysis by 'brown blotch' isolates. In contrast to the results of Hifni *et al.* (1975), we were unable to demonstrate 2-ketogluconate formation from sodium D-gluconate by resting cells even after 7 d. Hifni *et al.* (1975) used Benedict's reagent to detect reducing substances, whereas we used *o*-phenylenediamine-HCl reagent, which yielded a typical blue fluorescent spot with 2-ketogluconic acid under UV light. This reagent is sensitive up to 10 μg 2-ketogluconate (Gosselé *et al.*, 1980).

According to Reddy & Ou (1974), phenylalanine deaminase, tyrosinase and β -glucosidase activities were absent in *X. campestris* pv. *oryzae* and occurred in *X. campestris* pv. *oryzicola*. In our hands, however, the differentiating value of these tests is not so obvious. Phenylalanine deaminase activity was present in 53% of the *X. campestris* pv. *oryzicola* strains, but lacking in all strains from phenon 1 and phenon 3. Tyrosinase activity was only obvious in one strain, i.e. *X. campestris* pv. *oryzicola* NCPPB 1632. Discrepancies with the results of Reddy & Ou (1974) were also found for β -glucosidase activity with arbutin as substrate, as 91% of the pv. *oryzae* strains were able to hydrolyse arbutin, whereas only 67% of the pv. *oryzicola* strains were able to do so.

All *X. campestris* pv. *oryzae* and pv. *oryzicola* strains produced H_2S from L-cysteine and less abundantly from peptone. 'Brown blotch' isolates CIAT 1187 and CIAT 1191t2 did not produce H_2S from L-cysteine; none of the 'brown blotch' isolates formed H_2S from peptone.

The hydrolyse of Tweens 60 and 80 clearly differentiates phenons 1 and 2 (positive) from phenon 3 (negative). Hydrolysis of sodium polygalacturonate was only observed for a few strains, i.e. *X. campestris* pv. *oryzae* IRN 235, IRN 279, PDDCC 3124 and *X. campestris* pv. *oryzicola* NCPPB 1151, PDDCC 3131 and PDDCC 3136. There is no correlation between the pathogenicity towards rice variety TN1 and pectinolytic activity, confirming the findings of Hifni *et al.* (1975).

From Table 2 it is apparent that the 'brown blotch' isolates acidified a smaller number of carbohydrates than *X. campestris* pv. *oryzae* and pv. *oryzicola*. Acid formation from D-fructose was

evident after 24 h for the 'brown blotch' isolates, whereas many pv. *oryzae* and pv. *oryzicola* strains showed acidification only after 4 d. The absence of acid formation from D-galactose, cellobiose and trehalose clearly differentiates the 'brown blotch' isolates from pv. *oryzae* and pv. *oryzicola*. This confirms the data of Lozano *et al.* (1981). There are no striking contradictions between our results on acid formation by *X. campestris* pv. *oryzae* and those obtained by Goto (1964), Mukoo & Isaka (1964), Shekhawat & Srivastava (1968), Hifni *et al.* (1975), Reddy & Ou (1976) and Tsuchiya *et al.* (1982a).

The majority of the *X. campestris* pv. *oryzae* and pv. *oryzicola* strains were able to grow on D-xylose, D-glucose, D-galactose, D-fructose, cellobiose, sucrose, trehalose, sodium L-lactate and the Krebs' cycle intermediates sodium fumarate, sodium L-malate, sodium oxaloacetate and sodium succinate. (Poly)alcohols, polyphenols and their glycosides, and DNA bases are not suitable carbon sources for the growth of isolates from 'brown blotch', *X. campestris* pv. *oryzae* or pv. *oryzicola* strains. Inhibition reactions may occur: e.g. benzoic acid, added to GYEA basal medium at 0.01% (w/v) inhibits growth of both *X. campestris* pv. *oryzae* and pv. *oryzicola*. The growth tests on D-xylose, sodium L-lactate, glycerol, sodium propionate and L-alanine are useful features for the differentiation between the three plant pathogens. The pH is of utmost importance in these growth tests: below pH 5.5, growth on D-xylose, D-galactose, cellobiose, sucrose and trehalose was significantly reduced. None of the strains was able to grow anaerobically. When the inoculated plates were again incubated in normal atmospheric conditions after 7 d anaerobic incubation, growth did not recommence.

Some antibiotics inhibited the growth of *X. campestris* pv. *oryzae* and pv. *oryzicola* strains at low concentrations: tetracycline.HCl, novobiocin or doxycycline at 0.001% (w/v), and chloramphenicol at 0.005% (w/v). The 'brown blotch' isolates were the only strains which were able to grow in the presence of 0.005% (w/v) methicillin. *Xanthomonas campestris* pv. *oryzicola* is on the whole more resistant to antibiotics than pv. *oryzae*. This is especially obvious in the case of 0.001% (w/v) ampicillin. Unlike the majority of the *X. campestris* pv. *oryzae* strains, *X. campestris* pv. *oryzae* IRN 235 is resistant to 0.005% (w/v) neomycin and 0.001% (w/v) streptomycin, ampicillin or amoxycillin, thus resembling more the *X. campestris* pv. *oryzicola* strains. Cupric nitrate added to GYEA basal medium at 0.001% (w/v), permits a clear-cut differentiation between *X. campestris* pv. *oryzae* (resistant) and pv. *oryzicola* (susceptible).

All *X. campestris* pv. *oryzicola* and 'brown blotch' strains grew on 0.2% vitamin-free Casamino acids without carbon source.

Taxonomy of X. campestris pv. *oryzae*, pv. *oryzicola* and 'brown blotch' isolates

An excellent overall correlation was found between the groupings obtained by numerical analysis of phenotypic features and of protein electrophoregrams (Table 1, Fig. 1). All the *X. campestris* pv. *oryzae* strains, except IRN 235, belonged in phenon 1 and the electrophoretic cluster 1; all the *X. campestris* pv. *oryzicola* strains grouped in phenon 2 and electrophoretic cluster 2; the 'brown blotch' isolates constituted a separate phenon 3 and electrophoretic cluster 3 (Fig. 1). The excellent agreement between the groupings obtained by totally different approaches means that the numerical data can be interpreted with confidence.

The *X. campestris* pv. *oryzae* and pv. *oryzicola* strains investigated fit well in the phenotype definition of the genus *Xanthomonas* given by Bradbury (1984). The features of the strains from both pathovars correspond to those of *X. campestris* as given by Dye & Lelliott (1974) and Bradbury (1984). Only one aberration, i.e. the absence of growth at 35 °C, was found for both pathovars. Furthermore, the mol% G + C values of the DNAs of two *X. campestris* pv. *oryzae* strains (64.5 and 64.7 mol% G + C) and one *X. campestris* pv. *oryzicola* strain (65.0 mol% G + C) fit well in the mol% G + C range of 63.5 to 69.2 reported for the *Xanthomonas campestris* group (Bradbury, 1984). Murata & Starr (1973) determined mol% G + C values for *X. campestris* pv. *oryzae* of 64.4 and for *X. campestris* pv. *oryzicola* of 68.9. DNA:rRNA hybridizations (Table 3, Fig. 3) confirmed that *X. campestris* pv. *oryzae* and *X. campestris* pv. *oryzicola* belong to *Xanthomonas*. De Vos & De Ley (1983) found that this genus constitutes a separate rRNA branch of the second rRNA superfamily, together with the rRNA branches of *Pseudomonas fluorescens*,

Fig. 3. Position of *Xanthomonas campestris* pv. *oryzae*, *X. campestris* pv. *oryzicola* and the 'brown blotch' isolates in the second and fourth rRNA superfamilies. The rRNA cistron similarities are expressed as $T_{m(e)}$ values of DNA:rRNA hybrids. Not all known finer divisions of the branches are represented. The solid bar indicates the extent (heterogeneity) of the genus *Xanthomonas*. The roman numerals indicate the roots of the rRNA superfamilies. Data were obtained from the following sources: De Smedt & De Ley (1977), De Ley *et al.* (1978), De Smedt *et al.* (1980), Gillis & De Ley (1980), Swings *et al.* (1980), Bauwens (1980), Bauwens & De Ley (1981), De Vos & De Ley (1983), Swings *et al.* (1983), Van Landschoot & De Ley (1983).

Azomonas, *Azotobacter*, *Frateruia* (De Smedt *et al.*, 1980; Swings *et al.*, 1980), and *Marinomonas* (Van Landschoot & De Ley, 1983) (Fig. 3).

Numerical analysis of phenotypic features and protein electrophoregrams indicated that the 'brown blotch' strains were considerably different from *X. campestris* pv. *oryzae* and pv. *oryzicola* (Figs 1 and 2). DNA:rRNA hybridizations unequivocally proved that the 'brown blotch' isolates are not members of the genus *Xanthomonas* (Table 3). The 'brown blotch' isolates belong on the rRNA branch of the misnamed *Flavobacterium capsulatum*. The following species or strains have $T_{m(e)}$ values in the vicinity of the 'brown blotch' isolates: *Pseudomonas paucimobilis*, *Flavobacterium devorans*, '*Pseudomonas azotocolligans*' ATCC 12417 [not on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980)], and '*Chromobacterium lividum*' strains NCTC 10590 and NCTC 10591 (Bauwens & De Ley, 1981). All these organisms have been generically misnamed. Further investigations are required to determine the degree of relatedness of the 'brown blotch' isolates to the above mentioned taxa. These are all members of the fourth rRNA superfamily (*sensu* De Ley, 1978), which is constituted by a number of genera such as *Acetobacter*, *Gluconobacter*, *Agrobacterium*, *Rhizobium*, *Zymomonas*, *Beijerinckia*, *Azospirillum*, *Spirillum*, *Rhodopseudomonas*, etc. (De Smedt & De Ley, 1977; Gillis & De Ley, 1980; De Smedt *et al.*, 1980; M. Gillis & J. De Ley, unpublished). It should be recalled here that *F. capsulatum* and *F. devorans* (both 65 mol% G + C) (Holmes *et al.*, 1984) are very different from the type species *Flavobacterium aquatile* (32 mol% G + C) and that the named flavobacteria are distributed over at least six taxonomically widely different rRNA branches (Bauwens, 1980; Bauwens & De Ley,

Table 4. *Differentiation between X. campestris pv. oryzae, X. campestris pv. oryzicola and 'brown blotch' isolates*

+, ≥90% of the strains gave positive reactions; -, <10% of the strains gave positive reactions.

	<i>X. campestris</i> <i>pv. oryzae</i>	<i>X. campestris</i> <i>pv. oryzicola</i>	'Brown blotch' isolates
Oxidase	-	-	+
Acetoin production	-	+	-
H ₂ S from peptone	+	+	-
Hydrolysis of Tween 60 and Tween 80	+	+	-
Acid from D-galactose, cellobiose and trehalose	+	+	-
Growth on sole carbon source:			
D-xylose or sodium L-lactate	+	+	-*
glycerol or sodium propionate	-	-	+
L-alanine	-	+	+
Resistance towards (% w/v):			
methicillin (0.005)	-	-	+
cupric nitrate (0.001)	+	-	+
8-hydroxyquinoline (0.001)	-	-	+
zinc oxide (0.001)	-	-	+
Growth on 0.2% vitamin-free Casamino acids	-	+	+

* Very weak reaction.

1981). The phenotypic features of the 'brown blotch' isolates correspond roughly to the description of *P. paucimobilis* by Holmes *et al.* (1977).

The phenotypic differentiation between *X. campestris pv. oryzae*, *X. campestris pv. oryzicola* and the 'brown blotch' isolates is given in Table 4.

The fine taxonomic structure of X. campestris pv. oryzae

No phenotypic features were found allowing a subdivision of *X. campestris pv. oryzae*. This taxon is also fairly homogeneous on the basis of the protein electrophoregrams (Figs 1 and 2). In the phenotypic analysis two strains (PXO 61 and NCPPB 1152) clustered at a lower level in phenon 1, and strain IRN 235 grouped at the border of phenon 2. The latter strain occupied a separate position according to its protein electrophoretic pattern (Figs 1 and 2). However, this strain causes bacterial blight symptoms, typical of *X. campestris pv. oryzae* (Table 1). Hifni *et al.* (1975), Reddy & Ou (1976) and Tsuchiya *et al.* (1983a) were likewise not able to define biochemical groups within *X. campestris pv. oryzae*. The geographical distribution of the strains was not reflected in the dendrogram. Highly virulent and weakly virulent strains were intertwined in both dendrograms. The protein electrophoregram of the highly virulent strain IRN 325 (isolated in Indonesia) was almost indistinguishable from the pattern of the less virulent strain NCPPB 1153 (isolated in Japan) (Table 1; Fig. 2).

Classification systems for *X. campestris pv. oryzae* strains, based on their virulence on rice varieties bearing different major genes for resistance towards bacterial blight, have been developed in Japan (Ezuka & Sakaguchi, 1978) and in the Philippines (Mew & Vera Cruz, 1979), and five and four pathogenic groups, respectively, have been proposed within *X. campestris pv. oryzae*. In our phenotypic and gel electrophoretic analyses, we included representative strains of both systems of pathogenic groups: the Philippine groups I, II, III and IV were represented respectively by strains PXO 61, PXO 86, PXO 79 and PXO 71, whereas strains T7174, T7147, T7133 and H75304 were chosen from the Japanese pathogenic groups I, II, III and V, respectively. No representative of group IV was available. It can be concluded from Fig. 1 that there is no correlation between the clustering obtained from phenotypic analyses with S_{SM} or S_j coefficients and the pathogenic races of *X. campestris pv. oryzae*. No biochemical or physiological features were found that differentiated the pathogenic groups or correlated with the virulence of the strains.

This confirms the conclusions of Reddy & Ou (1976) and Tsuchiya *et al.* (1982a). Also, the protein electrophoregrams of strains PXO 61, PXO 86, PXO 79 and PXO 71 (Fig. 2) were very similar, suggesting a high degree of relatedness between the Philippine groups I, II, III and IV. Bacteria displaying a strong similarity in their electrophoretic protein patterns are known to share a high degree of DNA relatedness (Kerstens & De Ley, 1980; Izard *et al.*, 1981; Owen & Jackman, 1982). We can therefore conclude that all the investigated strains of *X. campestris* pv. *oryzae*, except strain IRN 235, constitute a genetically homogeneous group of bacteria, and that the subdivision in pathogenic races is determined by only a minute fraction of the genetic material of the cell.

The fine taxonomic structure of X. campestris pv. *oryzicola*

Within the *X. campestris* pv. *oryzicola* group all strains isolated in India by G. S. Shekhawat in 1966 and 1967 formed a small phenotypic subgroup, whereas strains PDDCC 3135 and PDDCC 3136, isolated in India in 1968, clustered with the other *X. campestris* pv. *oryzicola* strains isolated in the Philippines and Malaysia (Fig. 1a). The protein electrophoregrams revealed that all the strains isolated in India (including PDDCC 3135 and PDDCC 3136) shared a common heavy protein band at 44 mm from the top of the gel (Fig. 2). This common characteristic is reflected in the numerical analysis of the patterns (Fig. 1b). There is no correlation between the virulence of the strains and their position in the dendrogram or with any phenotypic feature. The protein patterns of the highly virulent strain NCPPB 1585 (neopathotype strain, isolated in Malaysia) and the non-virulent strain PDDCC 1622 (isolated in the Philippines) were very similar (Fig. 2). It has to be mentioned that within *X. campestris* pv. *oryzicola* no pathogenic races could be distinguished (Ou *et al.*, 1971).

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