Demonstration of synonymy between the plant pathogens *Pseudomonas avenae* and *Pseudomonas rubrilineans*

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Strains of *Pseudomonas avenae* Manns 1909 and *Pseudomonas rubrilineans* (Lee *et al.*, 1925) Stapp 1928 were compared using physiological, biochemical and serological tests together with an examination of plant host range. Minor differences were found between the physiological and biochemical characteristics of the two pathogens, while no differences were detected in pathogenicity, host range, cellular protein profiles, direct fluorescent staining, and dot-immunobinding assays. *P. avenae* and *P. rubrilineans* were distinctly different from other non-fluorescent pseudomonads. We were unable to differentiate *P. avenae* strains from those of *P. rubrilineans*, and we propose that they be regarded as a single species, retaining the name *Pseudomonas avenae*.

Introduction

During a survey of pearl millet (*Pennisetum glaucum*) in northern Nigeria in 1984, a bacterial leaf disease was observed in nearly every planting (Claflin *et al.*, 1989). Lesions, 3–25 cm in length, were usually confined within the leaf veins. Diseased leaf tissue yielded bacterial colonies on yeast extract/glucose/calcium carbonate agar medium (YDCA) (Dowson, 1957) and King's medium B (KB) (King *et al.*, 1954), which were smooth, cream coloured, and non-fluorescent. The colonies closely resembled those of *Pseudomonas avenae* (Manns, 1909) (syn. *P. alboprecipitans*; Schaad *et al.*, 1975). To identify the causal agent, we examined numerous other *Pseudomonas* spp; one of these, *P. rubrilineans*, the causal agent of red stripe in sugarcane (*Saccharum officinarum*) (Christopher & Edgerton, 1930; Hayward, 1962; Lee *et al.*, 1925), appeared to be similar to *P. avenae*.

In the work described here, the extent of this apparent similarity was determined by applying a range of criteria to nine isolates of *P. avenae* and 11 isolates of *P. rubrilineans*.

Methods

Bacterial strains and media. The strains used in this study are listed in Table 1. In addition to *P. avenae* and *P. rubrilineans*, strains of the non-

fluorescent plant pathogens *P. andropogonis* and *P. rubrisubalbicans*, and the fluorescent *P. syringae* pv. *syringae*, were studied. Stock cultures were maintained on plates of YDCA at 4 °C. Strains were lyophilized for long-term storage. Prior to each experiment, inocula from stock culture plates were restreaked on fresh YDCA and incubated for 24 h at 28 °C.

Biochemical and physiological tests. Arginine dehydrolase activity was determined by stabbing a culture into Thornley's medium 2A (Thornley, 1960) and recorded as positive if the medium turned red under anaerobic conditions. Media for determining malonate and citrate utilization, gelatin hydrolysis, oxidase production, and reduction of nitrate to nitrite, were obtained from Difco or made up according to the Difco Manual. Lipase activity was determined by the method of Sierra (1957). Hydrolysis of starch was determined by adding 2% (w/v) soluble potato starch (Sigma) to nutrient agar (Difco) supplemented with yeast extract (5 g l⁻¹). Inoculated plates were incubated for 4 d at 28 °C, and then flooded with 1% (v/v) Lugol's iodine. Growth at 4 °C and 41 °C was determined by incubating cultures on YDCA for a maximum of one month. Plates were examined weekly and scored positive if isolated single colonies were observed. Plates incubated at 41 °C were wrapped in Parafilm and placed in a moisture chamber to prevent media from dehydrating.

Acid production from various carbohydrate and carbon sources was tested using the OF basal medium of Hugh & Leifson (1953) (Difco) supplemented with the test compound at 1% (w/v). Acid production was scored positive if a colour change from green to yellow was observed throughout the length of the tube.

Hypersensitivity reactions were determined on tobacco (*Nicotiana tabacum* L. cv. 'Samsum') plants, essentially as described by Klement *et al.* (1964). Strains were grown overnight in nutrient broth (NB) (Vidaver, 1967), and the cultures centrifuged at 13000 g for 10 min. The pellets were resuspended in 12.5 mM-potassium phosphate buffer pH 7.1, centrifuged, washed two additional times in the same buffer, and adjusted to a cell concentration of 100 Klett units (Klett–Summerson filter no. 66) (about 10⁷ cells ml⁻¹). Cell suspensions were

Abbreviation: FITC, fluorescein isothiocyanate.

Table 1. *Origins, designations and hosts of Pseudomonas strains used in this study*

<i>Pseudomonas</i> strain*	Plant host	Origin
<i>P. avenae</i>		
ATCC 19860 ^T	<i>Zea mays</i>	Florida
ICPB PA134	<i>Zea mays</i>	Japan
ICPB PA135	<i>Zea mays</i>	Japan
ICPB PA138	<i>Zea mexicana</i>	Japan
76-1	<i>Zea mays</i>	Florida
C-10	<i>Zea mays</i>	Georgia
VS-1	<i>Paspalum urvillei</i>	Florida
ATCC 19822	<i>Oryza sativa</i>	Japan
W-6-L	<i>Pennisetum glaucum</i>	Nigeria
<i>P. andropogonis</i>		
ATCC 23061 ^T	<i>Sorghum bicolor</i>	Indiana
ATCC 23062	<i>Zea mays</i>	Indiana
KS 74	<i>Sorghum bicolor</i>	Kansas
Tx-5	<i>Sorghum bicolor</i>	Texas
PDDCC 6780	<i>Coffea arabica</i>	Brazil
<i>P. rubrilineans</i>		
ATCC 19307 ^T	<i>Saccharum officinarum</i>	Reunion
NCPBP 522	<i>Saccharum officinarum</i>	Mauritius
NCPBP 931	<i>Saccharum officinarum</i>	Australia
NCPBP 3029	<i>Zea mays</i>	Brazil
NCPBP 3107	<i>Zea mays</i>	Brazil
NCPBP 3111	<i>Canna paniculata</i>	Brazil
NCPBP 3112	<i>Canna indica</i>	Brazil
NCPBP 3113	<i>Saccharum officinarum</i>	Brazil
PDDCC 1656	<i>Zea mays</i>	Florida
PDDCC 3106	<i>Zea mays</i>	India
PDDCC 3168	<i>Zea mexicana</i>	India
<i>P. rubrisubalbicans</i>		
ATCC 19308 ^T	<i>Saccharum officinarum</i>	United States
PDDCC 793	<i>Saccharum officinarum</i>	Australia
PDDCC 2850	<i>Zea mays</i>	Florida
PDDCC 3109	<i>Saccharum officinarum</i>	Sri Lanka
PDDCC 6268	<i>Sorghum bicolor</i>	New Zealand
<i>P. syringae</i>		
pv. <i>syringae</i>		
ICPB 146N	<i>Sorghum vulgare</i> var. <i>sudanense</i>	Hungary

* ATCC, American Type Culture Collection, Rockville, MD, USA; NCPBP, National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire, UK; PDDCC, Plant Disease Division Culture Collection, Auckland, New Zealand; ICPB, M. P. Starr, International Collection of Plant Pathogenic Bacteria, Davis, CA, USA; 76-1, VS-1, R. E. Stall, University of Florida, Gainesville, FL, USA; C-10, N. W. Schaad, University of Georgia, Experiment, GA, USA; W-6-L, KS-74, Tx-5, Department of Plant Pathology, Kansas State University, Manhattan, KS, USA. A superscript T denotes a type strain.

infiltrated into the abaxial side of a mature tobacco leaf with a syringe until an area was water-soaked. Plants were maintained within a temperature range of 22–30 °C with a minimum of 12 h of light; observations were made 24 h after infiltration.

Pathogenicity tests. Pathogenicity of the various strains was determined on maize (*Zea mays*) cv. Pioneer 3195 (Pioneer Hi-Bred International, Johnston, IA, USA) and cv. Gold Cup (Harris-Moran Seed Co., Rochester, NY, USA); grain sorghum (*Sorghum bicolor*) cv. 80B3039 (D. T. Rosenow, Texas A & M University, Lubbock, TX, USA); pearl millet cv. Sidney Dwarf and cv. Serere 3A (D. J. Andrews, University of Nebraska, Lincoln, NE, USA); and sugarcane cv. 67500 (N. Zummo, Mississippi State University, Mississippi State, MS, USA). Four leaves of sugarcane were inoculated with each bacterial strain

using a Hagborg device (Hagborg, 1970) about 4 weeks after emergence. Five plants each of corn, sorghum, and pearl millet were inoculated with each strain about two weeks after emergence and maintained in a greenhouse (22–28 °C) with automated misting (6 min h⁻¹ for 6 h d⁻¹). Symptoms were recorded 2 weeks after inoculation.

Serological tests. *P. avenae* (ATCC 19860 and ICPB PA134), *P. rubrilineans* (ATCC 19307), *P. andropogonis* (ATCC 23061) and *P. rubrisubalbicans* (ATCC 19308) were used to produce antisera. The procedure was essentially as reported previously (Leach *et al.*, 1987) except that the rabbits were injected intramuscularly. Injections continued until serial twofold agglutination titres exceeded 1:2048. Antisera were fractionated twice using ammonium sulphate (Allan & Kelman, 1977), with a final concentration of 35% saturation. Fluorescein isothiocyanate (FITC) (Sigma) was used for conjugating the globulin fraction. Conjugation and direct staining were done by the methods of Allan & Kelman (1977), except that merthiolate was not added prior to storage. Microscope slides were examined using an Olympus BH-2 microscope equipped with a 40× Neofluor objective, an FITC (UG-1) exciter filter and an L420 barrier filter. The dot-immunobinding assay was performed as described previously (Leach *et al.*, 1987).

Cellular protein profiles. Cells were grown overnight in 5 ml of NBY in a shaker bath at 28 °C. Cells were pelleted in a clinical centrifuge, resuspended in 20 ml of cellular protein isolation buffer [10 mM-Tris hydrochloride, 30 mM-NaCl, pH 7.2 (Sigma)], and maintained on ice for the remainder of the procedure. A quantity of cells equal to 1300 Klett units [Klett reading × volume of suspension (ml) = 1300 Klett units] was centrifuged and resuspended in 1 ml isolation buffer and 1 ml 2 × sample buffer (2 × sample buffer: 1.51 g Tris hydrochloride, 20 ml glycerol, 4.0 g SDS, 10 ml mercaptoethanol, 20 mg bromophenol blue, 100 ml distilled water). Samples were boiled for 5 min, cooled, and stored at –20 °C. The buffer system for gel electrophoresis was that of Laemmli (1970). Polyacrylamide gel concentration and the procedure for protein band separation were as described by Qhobela & Claffin (1988).

Results

Phenotypic characteristics

Differences between *P. andropogonis*, *P. avenae*, *P. rubrilineans* and *P. rubrisubalbicans* are shown in Table 2. All four species were positive for acid production from arabinose, fructose and sorbitol, and for utilization of citrate, were positive for tobacco hypersensitivity, and grew at 4 °C. None produced acid from cellobiose, melibiose or salicin.

Serological tests

P. avenae and *P. rubrilineans* could not be distinguished from one another by the dot-immunobinding assay using heterologous antiserum at the highest dilution of 10⁵ (approximately 400 cells) (Table 3). Cross-reactions were observed with *P. andropogonis* and *P. rubrisubalbicans* at lower dilutions (4000 cells) to both antisera. This cross-reactivity was eliminated by decreasing the cell concentration. *P. andropogonis* and *P. rubrisubalbicans* reacted only with their homologous antisera at the highest dilutions. Neither *P. avenae* nor *P. rubrilineans* reacted with the antisera to the other pseudomonads.

Table 2. *Physiological and biochemical differences between P. andropogonis, P. avenae, P. rubrilineans and P. rubrisubalbicans*

Symbols: +, positive; -, negative; S, slight; ND, not done. Numbers in parentheses indicate the number of strains giving that response when the response varied among strains of one species.

Characteristic	<i>P. andropogonis</i> (4 strains)*	<i>P. avenae</i> (8 strains)†	<i>P. rubrilineans</i> (11 strains)	<i>P. rubrisubalbicans</i> (5 strains)
<i>Acid production from:</i>				
Glucose	-	+	+	+
Glycerol	-	+	+	+
myo-Inositol	+	-	-(7)	-(2)
Raffinose	-	-	-(6)	-
Rhamnose	-	-	-(5)	-
<i>Hydrolysis of:</i>				
Starch	-	S	-	-
Tween 80	ND	+	+	-
Growth at 41 °C	-	+	+	+
Utilization of malonate	+	+	-	+
Nitrate reduction	ND	+	+	-(2)
<i>Production of:</i>				
Arginine dehydrolase	-	-	-	+
Lipase	-	+	+	-
Oxidase	-	+	+	+

* ATCC 23061, ATCC 23062, KS 74, Tx-5.

† ATCC 19860, ICPB PA134, ICPB PA135, ICPB PA138, 76-1, VS-1, ATCC 19822, W-6-L.

Table 3. *Reaction of Pseudomonas strains to antisera prepared against four non-fluorescent pseudomonads*

+, Positive reaction in the dot-immunobinding assay at 10^5 c.f.u. ml⁻¹; -, negative reaction at 10^5 c.f.u. ml⁻¹.

Strain	Antiserum raised against:			
	<i>P. andropogonis</i>	<i>P. avenae</i>	<i>P. rubrilineans</i>	<i>P. rubrisubalbicans</i>
<i>P. andropogonis</i> ATCC 23061	+	-	-	-
<i>P. avenae</i> ATCC 19860	-	+	+	-
<i>P. rubrilineans</i> ATCC 19307	-	+	+	-
<i>P. rubrisubalbicans</i> ATCC 19308	-	-	-	+
<i>P. syringae</i> pv. <i>syringae</i> ICPB 146N	-	-	-	-
Water control	-	-	-	-

Immunofluorescent staining

In the direct staining procedure, *P. avenae* cells stained positive when incubated with FITC-conjugated antiserum to *P. rubrilineans*, at an optimal dilution of 1:64. Results were also positive when *P. rubrilineans* cells were incubated with FITC-conjugated antiserum to *P. avenae*. However, fluorescence was not observed when the direct staining procedure was used with cells of *P. andropogonis*, *P. rubrisubalbicans* or *P. syringae* pv. *syringae* and antiserum to *P. avenae* or *P. rubrilineans*.

SDS-PAGE

The protein banding patterns (Fig. 1) of *P. avenae* (lane G) and *P. rubrilineans* (lanes C and D) were indistinguishable from one another but clearly distinct from those of the other pseudomonads tested. *P. avenae* and *P.*

rubrilineans had a very large, distinct band at 29 kDa, along with other common bands, which were not observed for the other pseudomonads tested.

Pathogenicity tests

All the strains of *P. avenae* and *P. rubrilineans* tested were pathogenic to maize, millet and sorghum, and with the exception of strain NCPPB 3112 of *P. rubrilineans*, all were pathogenic to sugarcane. The plant disease symptoms induced by *P. avenae* and *P. rubrilineans* were indistinguishable. On maize, water-soaked areas were often present around the lesions, which had translucent to light centres with dark brown margins. Millet lesions were light brown at the centre with dark brown margins; they were confined to interveinal tissue and extended up to 10 cm. Water-soaking was limited to the edge of the margins. Sorghum lesions were tan-centred with dark red margins, while leaf veins near the margins were dark red.

Fig. 1. SDS-PAGE of cellular proteins from *Pseudomonas* strains. Lanes: A, *P. syringae* pv. *syringae* ICPB 146N; B, *P. andropogonis* ATCC 23061; C, D, *P. rubrilineans* ATCC 19307 and PDDCC 3168; E, F, *P. rubrisubalbicans* PDDCC 3109 and PDDCC 793; G, *P. avenae* ATCC 19860; H, molecular mass markers (SDS-7 Dalton VII-L).

On sugarcane, elongated dark red lesions were intertwined and extended up to 45 cm in length.

Our strains of *P. andropogonis* were highly aggressive in maize and sorghum, mildly virulent in sugarcane and weakly aggressive in millet. Strains of *P. rubrisubalbicans* incited a variable pathogenic response in corn and sugarcane and were non-pathogenic or caused a hypersensitive reaction in millet and sorghum.

Discussion

P. avenae and *P. rubrilineans* are presently regarded as separate species, but our phenotypic tests showed only minor differences between them. *P. avenae* was positive for malonate utilization whereas *P. rubrilineans* was negative. The ability to produce acid from *myo*-inositol,

raffinose and rhamnose was variable among the strains of *P. rubrilineans* and negative for all strains of *P. avenae*.

Twelve of the phenotypic characteristics tested were compared with those reported for *P. rubrilineans* (Hayward, 1962) and *P. avenae* (Schaad *et al.*, 1975). Our results for *P. rubrilineans* were the same as those obtained by Hayward (1962). However, our strains of *P. avenae* were oxidase positive whereas Schaad *et al.* (1975) reported theirs to be oxidase negative. The ability of *P. avenae* to hydrolyse starch and gelatin was reported as slight or variable by Schaad *et al.* (1975), whereas our results were negative. However, *P. andropogonis*, the cause of leaf spots and streaks in numerous monocot and dicot hosts (Moffett *et al.*, 1986), and *P. rubrisubalbicans*, a bacterium closely related to *P. rubrilineans* and the cause of mottled stripe on sugarcane (Christopher & Edgerton, 1930) and leaf stripe of sorghum (Hale & Wilkie, 1972), also exhibited only minor differences when compared to *P. avenae* and *P. rubrilineans*. Differentiation of species based solely on physiological and biochemical properties has always been difficult and, at times, subjective. The table of 16 different non-fluorescent pseudomonads given by Hayward (1983) exemplifies the problems encountered when differentiating bacterial species solely on the basis of such properties. While these properties are important and useful, other factors such as host range, symptomatology, serology, and comparison of protein profiles should be evaluated before taxonomic groupings are made.

Host range studies have reported *P. avenae* as naturally occurring on a wide variety of hosts including oats, barley, sorghum and wheat. *P. rubrilineans* has been associated mainly with sugarcane and sorghum. Both have been reported as being distributed worldwide. Our greenhouse studies failed to distinguish the two by host range or symptomatology on maize, sorghum, millet and wheat. The International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975) defines the pathovar as comprising bacteria that differ in 'pathogenic reactions in one or more hosts'. Since the symptomatology and host ranges were the same with *P. avenae* and *P. rubrilineans*, a pathovar designation would be invalid.

Dot-immunobinding assay procedures have proved both rapid and reliable in the identification of bacteria and viruses (Hsu, 1984; Leach *et al.*, 1987). *P. avenae* and *P. rubrilineans* reacted equally well with both homologous and heterologous antisera, while *P. andropogonis*, *P. rubrisubalbicans* and *P. syringae* pv. *syringae* were positive with their homologous antisera only. Additional serological evidence for the apparent synonymy of *P. avenae* and *P. rubrilineans* was provided by their equivalent reactions to homologous and heterologous FITC-conjugated antisera in direct immunofluorescent staining.

SDS-PAGE has been a valuable technique in bacterial identification and classification, and, at times, for the realignment of bacterial genera. Carlson & Vidaver (1982) used gel analysis as a major factor in the taxonomic reclassification of the genus *Corynebacterium*. In our work, the total cellular protein profiles of *P. avenae* and *P. rubrilineans* were indistinguishable from each other and formed a distinct group when compared to other non-fluorescent pseudomonads.

P. avenae and *P. rubrilineans* have consistently been placed in the same taxonomic group. De Vos *et al.* (1985) assigned *P. avenae* (*alboprecipitans*) and *P. rubrilineans* to the *P. acidovorans* rRNA branch. Takikawa & Shimaoka (1988) placed *P. avenae* in group C, which included *P. acidovorans* and *Comamonas terriagena*, on the basis of quinone and fatty acid profiles. Our results demonstrate that *P. avenae* and *P. rubrilineans* are synonymous. The name *Pseudomonas avenae* has priority and we propose that it be retained, with ATCC 19860 (NCPFB 1011) as the type strain.

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