Bacterial Diseases of Rice. II. Characterization of Pathogenic Bacteria Associated with Sheath Rot Complex and Grain Discoloration of Rice in the Philippines

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ABSTRACT

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From over 5,600 bacteria isolated from rice plants with sheath rot complex and grain discoloration syndrome, and two batches of 1 kg of rice seed (cultivars IR54 and IR8866), 204 pathogens were initially characterized by phenotypic tests, serology, and growth on selective media, and further distinguished by API 20NE, Biolog, and cellular fatty acid methyl ester-fingerprints. The best differentiation was obtained by the Biolog system. The nonfluorescent pathogens were represented by clusters D1 (*Burkholderia glumae*, formerly *Pseudomonas glumae*) and E (*Acidovorax avenae* subsp. *avenae*, formerly *Pseudomonas avenae*). Seven clusters were distinguished among the fluorescent strains associated with sheath rot complex and grain discoloration. Cluster A5 was identified as *Pseudomonas aeruginosa*, and cluster B1 as *P. fuscovaginae*. Cluster B2 is related to *Pseudomonas aureofaciens, P. corrugata, P. fluorescens,* and *P. marginalis*. Clusters B1 and B2 were only slightly different. The strains identified as *P. fuscovaginae* were different from the type strains in 2-ketogluconate production.

Sheath rot complex and grain discoloration of rice (*Oryza sativa* L.) generally describes the disease syndrome involving a brown discoloration or rot of the flag leaf sheath and discoloration of the grain. Both fungi and bacteria (mainly *Pseudomonas*) are reported to be associated with the disease syndrome (3,12,16,17,33). Several fluorescent and nonfluorescent pathogenic *Pseudomonas* spp. have been isolated from rice plants with sheath rot complex and grain discoloration in Latin America, Africa, and Asia (5,13,20,33,36). *Pseudomonas syringae* pv. *oryzae*, causal agent of halo blight (9), and *P. plantarii*, reclassified as *Burkholderia plantarii* (29), causal agent of seedling blight (1), have been reported so far only in Japan. *Pseudomonas avenae*, reclassified as *Acidovorax avenae* subsp. *avenae* (31), causes bacterial stripe, also known as brown stripe, and has long been recorded in the tropics (16,26). *Pseudomonas glumae*, reclassified as *Burkholderia glumae* (29), the cause of both seedling rot (28) and grain rot (6), was first reported in Japan and later in other Asian countries. *Pseudomonas fuscovaginae*, which causes sheath brown rot, was first reported in Hokkaido, North Japan (25). The species *P. fuscovaginae* was of special interest, as it was isolated from seedlings issued from seeds of cold-tolerant rice cultivars supplied by IRRI (Philippines) to ISABU (Burundi) (5). Reports on the occurrence of this pathogen in tropical Asia were lacking.

A prior study described the survey that was conducted to sample bacterial pathogens associated with grain discoloration and leaf sheath rot syndromes throughout the major rice growing districts in the Philippines (3). Pathogenicity tests identified 204 pathogenic forms out of the total pool of collected strains. The symptoms caused by these 204 bacterial pathogens were indistinguishable based on symptomatology induced by artificial inoculation (3); hence, the etiology of the disease syndrome could not be determined from symptoms only. Various selective media are reported to distinguish the different pathogenic pseudomonads of rice (11,13,18,27,34). In this study, we attempt to apply and confirm the efficiency of these media. The characterization and identification of *Pseudomonas* spp. is a long process when determined from cytological, morphological, and biochemical characteristics. As more techniques become available to meet the need for fast, automated, and reliable identification of bacteria, e.g., the Biolog GN MicroPlate system, API 20NE, and cellular fatty acid methyl ester (FAME) analysis, we plan to compare the applicability of these techniques. Also, the suggested combination of diagnostic tests for identification of *P. fuscovaginae* by Rott et al. (19) was applied on the Philippine strains. The objectives of this study were to determine the bacterial pathogens associated with sheath rot complex and grain discoloration in the Philippines, clarify the frequency of occurrence of the different *Pseudomonas* spp. involved, evaluate the potential of different identification methods for identification of bacterial pathogens of rice, and investigate the prevalence of *P. fuscovaginae* in the Philippines as reported (5,36) but never confirmed.

MATERIALS AND METHODS

Isolation of bacteria and pathogenicity. A survey was conducted to sample bacterial pathogens associated with grain discoloration and leaf sheath rot complex syndromes throughout the major rice growing districts in the Philippines during the wet seasons of 1988 and 1989. Isolation of bacteria from rice plants with the sheath rot complex and grain discoloration was done as described in the previous study (3). Based on pathogenicity tests, 204 pathogenic forms out of the total pool of collected strains were identified (3). The symptoms caused by these pathogenic strains, using inoculation methods of injection at seedling and booting stages, were indistinguishable. All pathogenic strains were lyophilized. The type and reference strains were obtained from the LMG Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

Selective media. The two media for detection of *B. glumae*, S-PG and PPGA + 0.1% $CaCl_2$, were prepared as described, respectively, by Tsushima et al. (27) and Matsuda et al. (11). Medium KBS of Rott et al. (18), arginine differential medium of Zeigler and Alvarez (34), and Miyajima's selective medium (13) were used for detection of *P. fuscovaginae*.

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Serology. An antiserum for *P. fuscovaginae* "A" was supplied by K. Mijayima (13), whereas antisera to *B. glumae* NIAES 1169^T and *B. plantarii* NIAES 1723^T were supplied by K. Azegami (1). Serological relationships were observed by the Ouchterlony double diffusion method using standard procedures (10). The wells had a diameter of 4 mm, and the distance between the centers of the wells was 9 mm. The agar plates loaded with the antigens and antisera were placed in a humidified plastic box covered with plastic wrap and kept at room temperature.

Biochemical characteristics. The pathogenic strains were divided into two groups according to fluorescent pigmentation on King's medium B (8). One-day-old nutrient agar cultures were used for the Gram staining, Kovac's oxidase reaction, and the inoculation of Hugh and Leifson's OF medium (4). Nitrate reduction, levan from sucrose, arginine dihydrolase, and 2-ketogluconate production were determined according to the methods described by Schaad (22) and Lelliot and Stead (10). Acid production from sucrose, inositol, sorbitol, and trehalose was tested on the medium of Ayers et al. (10).

The API 20NE galleries were used according to the manufacturer's specifications (API Systems Bio Mérieux SA 69280 Marcy-l'Etoile, France). The results were recorded after 48 h incubation at 28°C. The Biolog GN microplates (Biolog Inc., 3447 Investment Blvd., Suite 3, Hayward, CA 94545) were inoculated with a bacterial suspension (OD at 590 nm was about 0.250). The plates were incubated at 30°C for 48 h. Results were analyzed with Biolog GN database version 2.00 to determine the identity of each strain. Numerical analysis was performed using the simple matching coefficient and unweighted arithmetic average clustering. Growth conditions of cultures and preparation of the fatty acid methyl esters were performed according to Sasser (21). The generated profiles were analyzed using the Standard Aerobe Library provided by the Microbial Identification System (MIS version 3.2, Microbial ID Inc., Newark, DE). The same software was also used for unweighted, arithmetic average clustering and principal component analysis.

RESULTS

The 204 Philippine strains pathogenic for rice were aerobic Gram-negative rods (3). They were divided into two groups based on fluorescent pigmentation on King's medium B. The nonfluorescent group contained 19 strains, while 185 strains belonged to the diverse group of fluorescent pseudomonads. Twelve biochemical characteristics were determined for the 204 strains, as well as growth on 65 different carbon sources (results not shown). When the eight biochemical tests recommended for identification of *P. fuscovaginae* by Rott et al. (19) were used for comparison, none of the 204 Philippine strains entirely fitted the biochemical profile. The 11 strains identified by the Biolog system as *P. fuscovaginae* differed from Rott's *P. fuscovaginae* profile only for production of 2-ketogluconate (Table 1). Moreover, the biochemical profile of these 11 strains was unique among the 185 fluorescent strains. They were oxidase and arginine dehydrolase positive and produced acid from trehalose. They did not produce acids from inositol, sorbitol, or sucrose (with the exception of strain 4521). They were, however, positive for production of 2-ketogluconate. The biochemical profiles of the nonfluorescent strains identified by Biolog as *B. glumae* and *A. avenae* conformed to the phenotypic features of the species (1,2); there was a weak or positive Kovac's oxidase reaction for some of the *A. avenae* strains (1,35). The results obtained by Biolog, API, and FAME-MIS are shown in Table 2 for the type and reference strains and in Table 3 for the 204 rice strains.

Biolog. The 45 reference strains were correctly identified except for *B. glumae*, *B. plantarii*, *P. aureofaciens*, and the

Table 1. Selected biochemical characteristics of the strains pathogenic to rice identified by the Biolog GN MicroPlate system as *Acidovorax avenae*, *Burkholderia glumae*, and *Pseudomonas fuscovaginae*

IRRI number	Biolog identity	Biochemical tests[a]									
		NI	FL	ARG	KOV	LEV	SOR	SUC	INO	TRE	2-KG
1837	*A. avenae*	+[b]	–	–	–	–	+	–	–	–	–
1840	*A. avenae*	+	–	–	–	–	+	–	–	–	–
1845	*A. avenae*	+	–	–	–	–	+	–	–	–	–
1851	*A. avenae*	+	–	W	–	–	+	–	–	–	–
1891	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7010	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7012	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7014	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7015	*A. avenae*	+	–	W	+	–	+	–	–	–	–
7017	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7018	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7019	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7021	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7023	*A. avenae*	+	–	–	+	–	+	–	–	–	–
1857	*B. glumae*	+	–	–	W	–	+	–	+	+	–
1858	*B. glumae*	+	–	–	W	–	+	–	+	+	–
2056	*B. glumae*	+	–	–	–	–	+	–	+	+	–
2057	*B. glumae*	+	–	–	–	–	+	–	+	+	–
2076	*B. glumae*	+	–	–	–	–	+	–	+	+	–
4521	*P. fuscovaginae*	+	+	+	+	–	–	+	–	+	+
4605	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5793	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5801	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5803	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6031	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6202	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6235	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6609	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
7007	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
7008	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+

[a] NI = nitrate reduction test; FL = fluorescence on King's medium B; ARG = arginine dihydrolase test; KOV = Kovac's oxidase test; LEV = levan production from sucrose; SOR = acid production from sorbitol; SUC = acid production from sucrose; INO = acid production from inositol; TRE = acid production from trehalose; 2-KG = production of 2-ketogluconate.

[b] + = positive reaction; – = negative reaction; W = weak reaction.

pathovars of *P. syringae* (Table 2). The first two did not occur in the Biolog GN Data Base version 2. Table 3 shows the results of the identification of the 204 rice strains grouped according to the numerical analysis of the Biolog data. A simplified dendrogram of these results is represented in Figure 1. The majority of the rice strains associated with sheath rot and grain discoloration was grouped into five A clusters and two B clusters delineated at 90% similarity. The clusters A2, A3, A4, and A6 did not contain reference strains. Cluster A5 was related to *P. aeruginosa*. Cluster B1 contained 19 strains, including all *P. fuscovaginae* reference strains. Cluster B1, although displaying a unique phenotypic profile, was closely related to cluster B2, which contained 108 pathogenic rice strains and eight reference strains belonging to the species *P. marginalis*, *P. corrugata*, *P. fluorescens,* and *P. aureofaciens*. The homogeneous cluster D1 contained the *B. glumae* reference strains together with five strains; whereas cluster D2 contained the *B. plantarii* reference strains only. Cluster E comprised the *A. a.* subsp. *avenae* reference strains and 14 rice strains. The Biolog clustering reflected the phylogenetic relatedness between the different species involved in this study. All groups containing strains related to *P. fluorescens* (*Pseudomonas fluorescens* rRNA group of superfamily II) (30) or gamma subdivision (32) gathered into one cluster separate from the strains of rRNA superfamily III (30) or beta subdivision (32). The latter, comprising the former *P. glumae* and the former *P. plantarii,* belongs to the *solanacearum* rRNA complex, which has been transferred to the new genus *Burkholderia* (29), and *A. a.* subsp. *avenae* belongs to the *acidovorans* rRNA complex (31).

API. Many API codes either did not occur in the API profile index or identified the strain at genus level only (Tables 2 and 3). Strains with API code identical to the type or the reference strains were tentatively identified. This was the case for the strains belonging to *A. a.* subsp. *avenae* and *B. glumae* and for two strains related to *P. fuscovaginae*.

FAME-MIS. Identification of *A. a.* subsp. *avenae, B. glumae,* and *B. plantarii* was hampered by the absence of their profiles in the Standard Aerobe Library (version 3.2). Cluster analysis and principal component analysis revealed six homogeneous groups related to *P. syringae*, *P. aeruginosa*, *B. plantarii*, *A. a.* subsp. *avenae,* and (two groups) *B. glumae*. The mean fatty acid profiles of these groups are

Table 2. Identification and characterization of the type and reference strains

Type and reference strains	Biolog		API 20 NE	FAME-MIS
	Cluster	Identification (similarity)	Identification (profile)	Identification (similarity)
Pseudomonas aeruginosa LMG[a] 1242[T b]	A5	*P. aeruginosa* (0.689)	*P. aeruginosa* (1154575)	*P. aeruginosa* (0.758)
P. aureofaciens LMG 1245[T]	B2	*P. fluorescens* C (0.350)	*Pseudomonas* (1157557)	*P. aureofaciens* (0.700)
P. aureofaciens LMG 5832	B2		*Pseudomonas* (0157557)	*P. aureofaciens* (0.740)
Acidovorax avenae subsp. *avenae* LMG 1806	E	*A. avenae* (0.448)	No match (1205464)	*P. facilis* (0.550)
A. a. subsp. *avenae* LMG 2117[T]	E	*A . avenae* (0.534)	No match (1205454)	*P. facilis* (0.580)
A. a. subsp. *avenae* LMG 2118	E	*A. avenae* (0.501)	No match (1204464)	*P. facilis* (0.655)
A. a. subsp. *avenae* LMG 6516	E	*A. avenae* (0.665)	No match (1205464)	*P. facilis* (0.656)
A. a. subsp. *avenae* LMG 6517	E	*A. avenae* (0.680)	No match (1205460)	*P. facilis* (0.592)
A. a. subsp. *avenae* LMG 10904	E	*A. avenae* (0.835)	No match (1245474)	*P. facilis* (0.856)
P. corrugata LMG 2172[T]	B2	*P. corrugata* (0.765)	*Pseudomonas* (0057555)	
P. fluorescens LMG 1794[T]	B2	*P. fluorescens* A (0.612)	*P. fluorescens* (0147555)	*P. fluorescens* B (0.856)
P. fuscovaginae LMG 2158[T]	B1	*P. fuscovaginae* (0.642)	*Pseudomonas* (0157455)	*P. aureofaciens* (0.603)
P. fuscovaginae LMG 2192	B1	*P. fuscovaginae* (0.626)	*Pseudomonas* (0147455)	*P. aureofaciens* (0.353)
P. fuscovaginae LMG 5097	B1	*P. fuscovaginae* (0.627)	*Pseudomonas* (0147455)	*P. aureofaciens* (0.580)
P. fuscovaginae LMG 5742	B1	*P. fuscovaginae* (0.778)	*Pseudomonas* (0157455)	*P. aureofaciens* (0.320)
P. fuscovaginae LMG 12427	B1	*P. fuscovaginae* (0.825)	*P. fluorescens* (0146555)	*P. aureofaciens* (0.379)
P. fuscovaginae LMG 12428	B1	*P. fuscovaginae* (0.163)	*P. fluorescens* (0147555)	*P. putida* A (0.312)[c]
P. fuscovaginae LMG 12424	B1	*P. fuscovaginae* (0.501)	*Pseudomonas* (0047455)	*P. fluorescens* A (0.612)
P. fuscovaginae LMG 12425	B1	*P. fuscovaginae* (0.676)	*P. fluorescens* (0147555)	*P. aureofaciens* (0.360)
Burkholderia glumae LMG 1277	D1		No match (1477551)	No match
B. glumae LMG 2196[T]	D1		No match (1077051)	*B. cepacia* (0.748)
B. glumae LMG 10905	D1		No match (1077451)	*B. cepacia* (0.671)
B. glumae LMG 10906	D1		No match (1477451)	
P. marginalis pv. *marginalis* LMG 2210	B2	*P. marginalis* (0.756)	No match(0547555)	*P. fluorescens* C (0.531)
P. m. pv. *alfalfae* LMG 2214	B2		No match (1557555)	*P. fluorescens* C (0.806)
P. m. pv. *marginalis* LMG 2215	B2		*Pseudomonas* (1157555)	*P. fluorescens* A (0.670)
P. m. pv. *pastinacae* LMG 2238	B2	*P. marginalis* (0.700)	*Pseudomonas* (1157555)	*P. fluorescens* B (0.843)
B. plantarii LMG 9035[T]	D2	*B. caryophylli* (0.329)	*Pseudomonas* (1077555)	
B. plantarii LMG 10907	D2		*Pseudomonas* (0077555)	
B. plantarii LMG 10908	D2		No match (1077656)	*B. gladioli* (0.324)
B. plantarii LMG 10909	D2	*B. caryophylli* (0.325)	*Pseudomonas* (1077555)	
B. plantarii LMG 10910	D2		No match (1077554)	
B. plantarii LMG 10911	D2		No match (1077554)	
P. putida LMG 2257[T]	A1	*P. putida* B (0.798)	*P. putida* (0140457)	*P. aureofaciens* (0.321)
P. putida LMG 5835	A1	*P. putida* B (0.627)	*P. putida* (0140457)	
P. syringae pv. *oryzae* LMG 10912	C	*P. syringae* pv. *pisi* (0.451)	No match (0447477)	*P. syringae* (0.658)
P. s. pv. *oryzae* LMG 10913	C	*P. syringae* pv. *aptata* (0.569)	*P. chlororaphis* (0447451)	*P. syringae* (0.545)
P. s. pv. *oryzae* LMG 10914	C	*P. s.* pv. *aptata* (0.628)	*P. chlororaphis* (0447441)	*P. syringae* (0.595)
P. s. pv. *oryzae* LMG 10915	C	*P. s.* pv. *pisi* (0.405)	*P. chlororaphis* (0447441)	*P. syringae* (0.592)
P. s. pv. *oryzae* LMG 10916	C	*P. s.* pv. *aptata* (0.668)	No match (0447450)	*P. syringae* (0.314)
P. s. pv. *oryzae* LMG 10917	C	*P. s.* pv. *pisi* (0.451)	*P. chlororaphis* (0447451)	*P. syringae* (0.640)
P. s. pv. *oryzae* LMG 10918	C		*P. chlororaphis* (0447441)	*P. syringae* (0.599)
P. s. pv. *oryzae* LMG 10919	C	*P. s.* pv. *tabaci* (0.412)	*P. chlororaphis* (0447441)	*P. syringae* (0.394)
P. s. pv. *oryzae* LMG 10920	C	*P. s.* pv. *pisi* (0.322)	No match (0447440)	*P. syringae* (0.385)
P. syringae pv. *panici* LMG 2367	C	*P. s.* pv. *aptata* (0.547)	*P. chlororaphis* (0447455)	*P. syringae* (0.825)

[a] LMG = Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent.
[b] [T] Type strain.
[c] Second choice.

shown in Table 4. The majority of the strains and reference strains belonging to the fluorescent pseudomonads constituted a heterogeneous group. A mean profile for this group is also shown in Table 4. It is only indicative and should be considered with care. The hydroxy fatty acids have discriminative value (15,24). They constituted the only qualitative differences between the fluorescent pseudomonads and *P. syringae* and *P. aeruginosa* on one hand, and *B. glumae* and *B. plantarii* on the other hand. The hydroxy fatty acids 12:0 2-OH and 12:0 3-OH were characteristic of the former group, while 16:1 2-OH, 18:1 2-OH, and summed feature 3 were indicative of the latter. *A. a.* subsp. *avenae* contained fewer fatty acids but was characterized by significantly higher amounts of 16:1 *cis* 9. *B. glumae* and *B. plantarii* differed from each other in the amount of 17:0 cyclo, 19:0 cyclo, and summed feature 7. The differences between the two groups of *B. glumae* were only quantitative. The amount of the following fatty acids was significantly different among *P. syringae*, *P. aeruginosa,* and the other fluorescent pseudomonads: 12:0 2-OH, 12:0, 17:0 cyclo, 16:1 cis 9, 16:0 3-OH, and summed feature 7.

Ouchterlony double diffusion tests. None of the 11 strains resembling *P. fuscovaginae* reacted with the antiserum to *P. fuscovaginae* "A" provided by K. Mijayima. Antigenic variability from the Japanese strain may be the reason. Also, a negative serological reaction was previously reported by Rott et al. (20) as not exclusive for identification of *P. fuscovaginae*. Of the 19 nonfluorescent strains, only five (1857, 1858, 2056, 2057, and 2076) reacted with the antiserum to *B. glumae* NIAES 1169^T, and none formed precipitin bands with the antiserum to *B. plantarii* NIAES 1723^T (Table 5).

Selective media. An overview of the tested selective media described in the literature for differentiating bacterial pathogens related to sheath rot and grain discoloration is shown in Table 6. The diverse group of fluorescent strains, comprising 90% of the 204 collected pathogens, were all tested on *P. fuscovaginae*–selective media, since no other selective media for fluorescent rice pathogens were noted in the literature. Although KBS medium was suggested as a semiselective medium to isolate *P. fuscovaginae*, all fluorescent strains except seven grew on it. Also, 44 strains were tentatively identified as *P. fuscovaginae* on the arginine differential medium. Only the medium described by Miyajima proved to be more selective. Three strains (6202, 6235, 6031) showed the characteristic cream colonies with greenish center after incubation (Fig. 2). However, eight strains identified by Biolog as *P. fuscovaginae* did not show the typical colony morphology on this medium.

Differentiation of the nonfluorescent strains was made by use of selective media for *B. glumae*. No calcium oxalate crystals, a typical characteristic for *B. glumae*, were produced by any of the nonfluorescent strains after incubation on potato-peptone-glucose agar (PPGA) medium supplemented with 0.1% $CaCl_2$. Growth on the arginine differential medium (34) appeared not to conform with the identities obtained by the Ouchterlony double diffusion tests (ODD) and by Biolog, as shown in Table 5. On S-PG medium, both colony types A (reddish brown) and B (purplish pink) were observed. While colonies identified as *B. glumae* type B could actually be *A. a.* subsp. *avenae*, five strains (Table 5) were positively identified as *B. glumae* in combination with the ODD tests. This procedure was recommended by Tsushima et al. (27).

DISCUSSION

The previous study confirmed the presence of different bacterial pathogens associated with sheath rot complex and grain discoloration in the Philippines (3). Of the total number of strains collected from rice tissues with sheath rot complex and grain discoloration syndrome, only 3.6% was found pathogenic (3). In agreement with earlier reports, it is known that various pseudomonads may be isolated from rice plants displaying the disease syndrome, and that apart from the small proportion of pathogenic species, the majority of these bacteria are saprophytic (19,20,33,35). The symptoms caused by these strains

Table 3. Identification and characterization of the pathogenic strains associated with sheath rot complex and grain discoloration syndrome on rice

Number and origin of pathogenic strains[a]	Biolog		API 20 NE	FAME-MIS
	Cluster	Identification (similarity)[b]	Identification (profile)	Identification (similarity)[b]
1 seed isolate (IR54)	A2	*Pseudomonas fluorescens* C (0.682-0.862)	*Pseudomonas* (0156557)	*P. aureofaciens* (0.270-0.527)
2 ShR[c] & GD[d] isolates				
3 seed isolates (IR8866)	A3	*P. fluorescens* C (0.638-0.768)	*Pseudomonas* (1152555)	
22 seed isolates (IR54, IR8866)	A4	*P. fulva* (0.234-0.451)	No match (0054455)	*P. putida* A (0.181-0.755)
21 ShR & GD isolates		*P. putida* A (0.137-0.790)	*Pseudomonas* (0056451)	
			Pseudomonas (0056455)	
			Pseudomonas (0154455)	
			P. fluorescens (0156455)	
2 seed isolates (IR54, IR8866)	A5	*P. aeruginosa* (0.834-0.826)	*P. aeruginosa* (0154475)	*P. aeruginosa* (0.660-0.739)
2 Shr & GD isolates			*P. aeruginosa* (1154475)	
9 seed isolates (IR54, IR8866)	A6	*P. fluorescens* C (0.093-0.697)	*Pseudomonas* (0156457)	No match
4 ShR & GD isolates			*Pseudomonas* (0156557)	
2 seed isolates (IR8866)	B1	*P. fuscovaginae* (0.711-0.917)	*P. fluorescens* (0147555)	No match
9 ShR & GD isolates			*P. fluorescens* (0157455)	*P. putida* A (0.381)
			Pseudomonas (0157555)	
3 seed isolates (IR54, IR8866)	B2	*P. corrugata* (0.334-0.424)	*P. fluorescens* (0147555)	*P. aureofaciens* (0.135-0.796)
105 ShR & GD isolates		*P. marginalis* (0.090-0.838)	*P. fluorescens* (0156555	*P. fluorescens* B (0.106-0.802)
		P. fluorescens B (0.747)	*Pseudomonas* (0157555)	*P. putida* A (0.197-0.769)
			No match (0357555)	
5 ShR & GD isolates	D1		No match (1077051)	No match
			No match (1077451)	
			No match (1477551)	
14 seedling isolates	E	*Acidovorax avenae* (0.188-0.900)	No match (1205474)	*P. facilis* (0.145-0.509)
			No match (1205464)	*Hydrogenophaga pseudoflava* (0.123-0.457)
			No match (1205460)	
			No match (1247577)	

[a] Seed isolates were obtained from 1-kg seed batches.
[b] Minimum and maximum similarity is mentioned when more strains gave same identification.
[c] ShR = sheath rot.
[d] GD = grain discoloration.

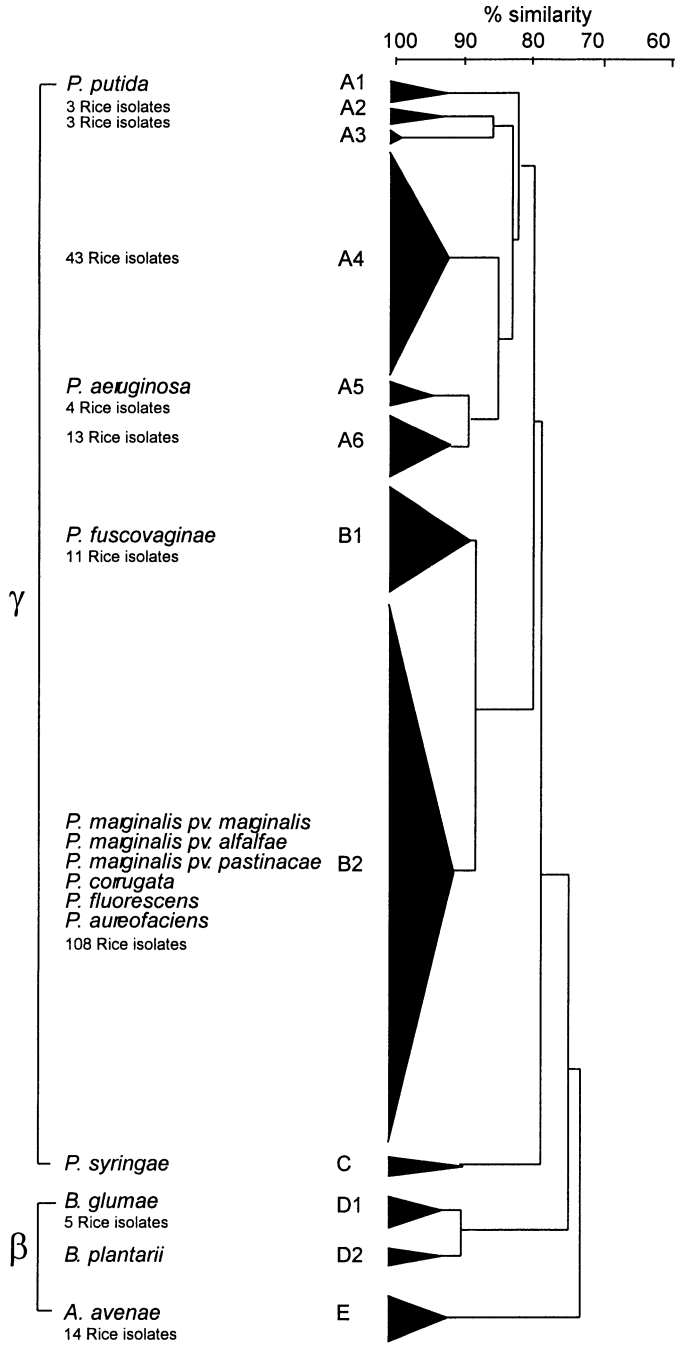

Fig. 1. Simplified dendrogram showing the Biolog clusters of the 204 pathogenic strains and 40 reference strains. The species names refer to the type strains and/or reference strains that were included. The designations β and γ refer to the subdivisions distinguished by Woese (32).

were similar (3), preventing determination of the etiology from symptoms alone. More than half of the 204 pathogenic strains were grouped by Biolog into cluster B2, together with eight reference strains of the species, *P. marginalis*, *P. corrugata*, *P. fluorescens,* and *P. aureofaciens*. The cluster B1 containing *P. fuscovaginae* produced lesions rapidly, but was indistinguishable from cluster B2 for the types of lesions produced (3). However, all strains of cluster B2 differed phenotypically from cluster B1 for at least three biochemical characteristics, generally being positive for acid production from inositol, sorbitol, and sucrose. Although these *Pseudomonas* spp. are usually regarded as saprophytes, their possible role in the development of the disease syndrome has not been recognized so far. They were, however, identified in this study as pathogenic forms able to induce leaf sheath browning. *P. marginalis* has been involved to some extent in sheath rot of rice (7,20,33,35). Therefore, we feel that their identity needs to be further clarified and established.

P. syringae was not detected among the Philippine strains. All phenotypical characteristics suggested the absence of this species, and none of the strains was identified as such by Biolog or FAME. Of the bacterial pathogens commonly reported to be involved in sheath rot complex and grain discoloration, three were encountered in this study. They were *P. fuscovaginae*, *B. glumae,* and *A. a.* subsp. *avenae*. However, they represented only 15% of the collected pathogenic strains associated with the disease syndrome in the Philippines. *A. a.* subsp. *avenae* strains that produced the brown stripe on the leaf midrib of inoculated seedlings were the only pathogens recognizable by a typical symptom expression in the pathogenicity tests (3). However, because it was isolated only from seedlings, the role of *A. a.* subsp. *avenae* in sheath rot complex and grain discoloration in the Philippines was not clear. Five strains were identified as *B. glumae* by the appearance of the typical colony formation on S-PG medium in combination with a positive serological reaction to *B. glumae* NIAES 1169^T antiserum, as was recommended by Tsushima et al. (27). They also fit the biochemical profile of the species (1,2) and were congruent with the Biolog identification. Although phenotypically related to *B. glumae* (1), none of the strains reacted with the *B. plantarii* NIAES 1723^T antiserum, and none produced the reddish brown pigment associated with tropolone production in the media (1). This suggested the absence of *B. plantarii* in the collected pathogens. The species *P. fuscovaginae* was of special interest, as it was suspected to occur in the Philippines (5,35); whereas a study on the occurrence of this pathogen in tropical Asia was lacking. *P. fuscovaginae* is reported to be widespread and is considered the principal causal organism of sheath browning and grain discoloration (35). It has been described by Miyajima et al. (14) and confirmed by Duveiller et al. (5) as a fluorescent pseudomonad that is positive for the arginine dehydrolase and oxidase reactions and that can be distinguished from other species within this group by the simultaneous lack of 2-ketogluconate production and presence of acid production from trehalose but not from inositol. Further, Rott et al. (19) reported that the combination of pathogenicity and a biochemical profile of eight characteristics permitted the identification of *P. fuscovaginae*, while a serological negative reaction was not exclusive. Several authors reported serological variability within the *P. fuscovaginae* species (5,19,20,35). From the 185 Philippine fluorescent pathogenic strains, only 11 strains were clustered with *P. fuscovaginae* by Biolog. All of these 11 strains were pathogenic on rice (3), and all except one conformed to seven of the eight characteristics in the biochemical profile of Rott et al. (19). However, they were positive for production of 2-ketogluconate (Table 1). Strain 4521 was different from the others in being positive for both nitrate reduction and acid production from su-

Table 4. Fatty acid profiles of the Biolog clusters

	A1,A2,A3,A4,A6,B1,B2[a]	C	A5	D1		D2	E
10:0 3-OH	6.0 (4.1)[b]	5.4 (1.0)	4.9 (0.5)	tr			6.2 (1.0)
12:0	1.5 (0.9)	5.3 (0.4)	3.2 (0.1)			3.4 (0.5)	
12:0 2-OH	7.2 (1.5)	3.4 (0.3)	4.5 (0.2)				
12:1 3-OH			0.4 (0.3)				
12:0 3-OH	5.5 (1.0)	4.9 (0.6)	4.5 (0.2)	tr			
14:0	0.3 (0.1)	tr	0.6 (0.1)	4.7 (0.7)	3.7 (0.0)	4.6 (0.3)	2.2 (0.5)
? 14.503				1.6 (1.2)			
15:0			tr			0.8 (1.5)	
16:1 *cis* 9	23.8 (5.9)	39.1 (0.8)	14.4 (3.5)	6.3 (2.6)	16.29 (2.9)	4.6 (1.2)	45.7 (2.1)
16:0	28.0 (3.3)	25.0 (1.2)	23.3 (1.6)	18.9 (3.8)	22.67 (0.2)	23.7 (2.0)	28.8 (2.0)
17:0 cyclo	7.4 (4.9)		1.0 (0.7)	10.61 (2.3)	3.6 (3.15)	20.0 (3.1)	
16:1 2-OH				tr	1.2 (0.0)	0.4 (0.4)	
16:0 2-OH				tr	0.9 (0.0)	tr	
16:0 3-OH		1.1 (0.8)		5.5 (0.2)	4.55 (0.0)	6.6 (0.6)	
18:0	0.7 (0.2)	0.6 (0.1)	0.5 (0.1)		1.1 (0.2)	0.8 (0.5)	
19:0 cyclo C11-12	0.4 (0.4)		1.5 (1.0)	6.4 (2.0)	2.4 (2.0)	10.0 (0.9)	
18:1 2-OH				4.7 (0.9)	3.0 (0.5)	5.1 (0.4)	
SUM 3[c]				13.7 (6)	5.4 (0.1)	8.3 (4.3)	
SUM 7[d]	16.2 (3.4)	14.4 (1.5)	38.3 (1.0)	25.5 (3.5)	34.7 (3.2)	14.6 (2.3)	12.3 (2.0)

[a] Referring to the Biolog clusters.
[b] Percent fatty acid; standard deviation in parenthesis.
[c] Summed features 16:1 *iso* and 14:0 3-OH.
[d] Summed features 18:1 *cis* 11 and 18:1 *trans* 11.

crose, which has also been reported for *P. fuscovaginae* strains from Colombia (33). On the medium of Miyajima, only three strains appeared positive by the appearance of the characteristic cream colonies with greenish center (Fig. 2). None of the 11 strains gave a positive serological reaction with the antiserum to *P. fuscovaginae* "A" from Miyajima, suggesting that the Philippine strains were serologically different from that of Japan.

Notwithstanding the use of three commercially available identification techniques—API Systems, Biolog GN MicroPlate System, and FAME-MIS—and the use of reference strains, only 23 pathogenic rice strains were unambiguously identified as either *P. aeruginosa*, *B. glumae*, or *A. a.* subsp. *avenae*. For the remaining 181 pathogens, the results were conflicting, although API and Biolog were comparable. However, these techniques provided an extensive characterization and constituted a real advantage, since a large number of characteristics could be checked in a short time. When identification was hampered by the lack of certain species in the library, cluster analysis of the data was very helpful. Identification was also determined by the type of characteristics used. The number of fatty acids produced by the species in this study varied around 10, apparently not always enough to allow differentiation. As demonstrated (17,24), they were not enough for differentiating most of the fluorescent pseudomonads.

Except for *P. syringae* and *P. aeruginosa*, differentiation of most bacteria was mainly based on metabolic activity. Nutritional characteristics have long been an important differentiating tool for this group of bacteria (23). The two identification systems based on metabolic activity, API and Biolog, matched well. Each Biolog cluster was characterized by a unique set of API codes, except for the closely related clusters B1 and B2, which both contained strains with the API 20NE codes 0147555 and 0157555.

Table 5. Characterization of the nonfluorescent pathogenic strains by selective media and serology

IRRI number	Biolog identity	SPG medium[a]	Arginine medium	Serological reaction[b] *B. glumae* NIAES 1169[T]	Serological reaction[b] *B. plantarii* NIAES 1723[T]
1857	*Burkholderia glumae*	*B. glumae* A	*B. glumae*	+	–
1858	*B. glumae*	*B. glumae* A	*B. glumae*	+	–
2056	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
2057	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
2076	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
1837	*Acidovorax avenae*	*B. glumae* B	*B. glumae*	–	–
1840	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1845	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1851	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1891	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7010	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7012	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7014	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7015	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7017	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7018	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7019	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7021	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7023	*A. avenae*	*B. glumae* B	*B. glumae*	–	–

[a] Type A colonies = reddish brown; type B colonies = opalescent purple.
[b] Ouchterlony double diffusion test (ODD) where + = formation of precipitin bands and – = absence of precipitin bands.

Although the pathogenic species reportedly associated with the disease syndrome are separately described in the literature as causing distinct symptoms, the differentiation between these symptoms was not clear. In addition, as might be expected, there are no symptoms described in the literature for the large group of encountered pseudomonad saprophytes. Hence, the etiology of sheath rot complex and grain discoloration syndrome could not be determined from symptoms alone. The use of selective media to differentiate the various pathogens proved to be of limited value. The characterization and identification of pseudomonads by conventional procedures of cytology, morphology, and biochemical characteristics is time-consuming and greatly hampered by the implicit complexity and problems associated with the present taxonomy of the pseudomonads. From the three automated systems (Biolog GN MicroPlate, FAME, and API) applied, the Biolog GN MicroPlate system in combination with cluster analy-

sis was the most helpful to differentiate the various pathogens. The Biolog library was, however, limited in the number of bacterial pathogens of rice. The efficiency of the system was improved by including several reference strains. Notwithstanding the application of the Biolog system with incorporation of several reference strains and cluster analysis of the data, the majority of the 204 strains still could not be identified accurately. Definitive phenotypic differentiation of these pathogenic strains may require the use of more than the 95 tests available in the Biolog GN MicroPlate system.

Surprisingly, considering that these Biolog groups were associated with sheath rot complex and grain discoloration in the Philippines, the most frequently isolated pathogens were either related to saprophytic *Pseudomonas* spp. or remained unidentified (3). Their identity and specific role in the development of the disease syndrome needs to be further established. Serological (19,20), as well as phenotypical, variability (5,35) within the species *P. fuscovaginae* has been reported, suggesting intraspecies differences and questioning its designation as a defined species. The Philippine strains, clustered with *P. fuscovaginae* by the Biolog GN MicroPlate system, were few in number. None produced the typical brown sheath symptoms described by Tanii et al. (25). Further, they were serologically different from the *P. fuscovaginae* strain of Miyajima, and differed phenotypically from Miyajima et al.'s (14) and Rott et al.'s (19) biochemical profile for 2-ketogluconate production. These results also are in contradiction with the characteristics earlier attributed to the *P. fuscovaginae* strains that were suspected to occur in the Philippines (5,35). As the cluster containing *P. fuscovaginae* was only slightly different from the group containing the majority of the fluorescent pseudomonads, including the type strains of *P. marginalis* and *P. fluorescens*, other techniques are needed to improve our insight into the genomic relatedness of these pathogens.

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Table 6. Evaluation of selective media for the detection of *Burkholderia glumae* and fluorescent pathogenic pseudomonads of rice

Group	Selective media	Diagnostic value
Nonfluorescent	S-PG medium for *B. glumae*	+[a]
	Potato-peptone-glucose agar (PPGA) with 0.1% $CaCl_2$ for *B. glumae*	–
Fluorescent	Miyajima's selective medium for *Pseudomonas fuscovaginae*	+
	KBS semiselective medium for *P. fuscovaginae*	–
	Arginine differential medium	–

[a] + = satisfactory; – = poor.

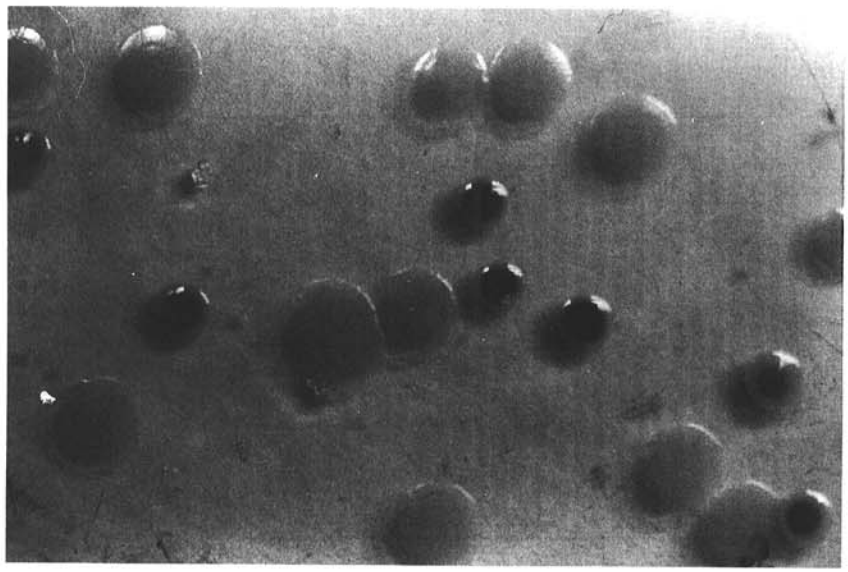

Fig. 2. Typical greenish colorations in the center of colonies of strain 6031 on the selective medium of Miyajima for *Pseudomonas fuscovaginae*.

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