Identification of *Pseudomonas fuscovaginae* with Biochemical, Serological, and Pathogenicity Tests

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ABSTRACT

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Three types of diagnostic tests were used to identify 34 strains of *Pseudomonas fuscovaginae* out of 136 strains of fluorescent pseudomonads isolated from diseased rice (*Oryza sativa*) samples from fields in five countries. Some of the remaining 102 strains that did not conform to *P. fuscovaginae* were pathogenic in rice. More than two serotypes existed among strains of *P. fuscovaginae* from Madagascar. Only the combination of pathogenicity and a biochemical profile, i.e., identical reactions to seven reference strains in eight biochemical tests, permitted identification of all strains of the pathogen.

Various fluorescent pseudomonads can be isolated from rice (*Oryza sativa* L.) plants with symptoms of grain discoloration and sheath rot. The majority of these bacteria are saprophytic; however, some, such as *Pseudomonas syringae* pv. *syringae* van Hall and *Pseudomonas fuscovaginae* Miyajima, Tanii, and Akita, are pathogenic (14). The latter species causes a relatively new disease known as sheath brown rot of rice, which was first identified in 1976 in Japan (11) and has since been found in Burundi (1,2), Rwanda (2), Zaire (3), Madagascar (3,10), and Latin America (12–14).

The characterization and identification of *P. fuscovaginae* is a relatively long process when determined from cytological, morphological, and biochemical characteristics. However, this bacterium can be distinguished from other fluorescent pseudomonads that are arginine dihydrolase and oxidase positive by a minimum set of several biochemical characteristics, including production of 2-ketogluconate, acid production from trehalose and inositol, and the utilization of inositol, sorbitol, and 2-ketogluconate (2,8). Additionally, antisera to *P. fuscovaginae* appear to be relatively specific, e.g., very few cross-reactive reactions were observed with other bacterial species although at least three serotypes exist (2,7,10,14). Therefore, serological techniques could contribute to the rapid detection and identification of the pathogen if antigenic variability within the species is taken into consideration (10).

The objective of this study was to determine if *P. fuscovaginae* could be reliably identified with a combination of biochemical tests, a serological technique, and a pathogenicity test.

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MATERIALS AND METHODS

Plant material. Plant material used for isolation of bacteria was collected in 1987 and 1988 and originated from Brazil, Cameroon, Madagascar, Reunion Island, and Rwanda. Plants with sheath rot symptoms were randomly sampled from different rice fields.

Media used. Three media were used for isolation of bacteria: King's Medium B (KB), medium KBS of Rott et al (9), and medium S1 of Gould et al (5). The latter was modified as follows: 10 g of sucrose, 10 ml of glycerol, 5 g of casamino acid (Difco Laboratories, Detroit, MI), 1 g of $NaHCO_3$, 1 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of K_2HPO_4, 18 g of agar, 20 mg of trimethoprim (Sigma Chemicals, St. Louis, MO), 50 mg of penicillin G (Sigma), 50 mg of bacitracin (Sigma), 100 mg of cetrimide (Labosi, Paris, France), and 50 mg of Actidione (cycloheximide) (Labosi). Trimethoprim, penicillin G, bacitracin, and Actidione were mixed together in 70% ethanol. A distilled water stock solution (20 mg/ml) was prepared for cetrimide. The antibiotics were added to a freshly autoclaved basal medium after it had cooled to about 50 C.

Isolation of bacteria. Rice grains that were healthy or exhibited symptoms of necrosis (about 1:1) were washed in running tap water for 1 hr and then placed directly on one of the three isolation media. Pieces of diseased sheath tissue were macerated directly in sterile distilled water. Loopfuls of macerated tissue were streaked onto KB. In 1987, isolations were also made on S1 medium, but this medium was replaced in 1988 by KBS because the morphology of *P. fuscovaginae* colonies was different on the S1 medium than on KB. Only fluorescent bacteria that appeared after 1–5 days were selected and subcultured. Usually, several colony types (color, shape, and growth rate) developed on isolation medium streaked with a single sample. Seven reference strains of *P. fuscovaginae* were used, including 6801, 7103,

Table 1. Pathogenicity and serological reaction of known strains of *Pseudomonas fuscovaginae*

Strain	Origin	Biochemical profile[a]	Pathogenicity[b] (no. of plants)			Tube agglutination[c] antiserum[d]	
			Without visible symptoms	With necrosis of foliar sheath	Dead	Strain GR2	Strain HMB266
6801	Japan	+	7	8	0	+	+
7103	Japan	+	2	13	0	−	+
BM1	Japan	+	6	9	0	−	+
HMB264	Burundi	+	1	14	0	+	+
BCE32	Colombia	+	2	13	0	−	−
532	Colombia	+	12	3	0	−	−
GR2	Madagascar	+	0	6	9	+	+

[a] Positive reaction for oxidase, arginine dihydrolase, and production of acids from trehalose; negative reaction for production of 2-ketogluconate, levan, and acids from inositol, sucrose, and sorbitol.
[b] Observed 20 days after inoculation. Each strain is inoculated on 15 3-wk-old plants of variety IRAT 13.
[c] + = Flocculent precipitate formation, − = absence of flocculent precipitate.
[d] Diluted to 1/200.

Table 2. Biochemical, pathological, and serological properties of 136 bacterial strains isolated from rice originated in Brazil, Cameroon, Madagascar, Reunion Island, and Rwanda

Biochemical characters[a]	Number of strains[b]				
	Total	P+ S+	P+ S−	P− S+	P− S−
0	36	24	10	0	2
1	18	0	0	0	18
2	36	0	0	0	36
3	12	0	0	0	12
4	2	0	0	0	2
5	29	0	22	3	4
6	3	0	2	0	1
7 or 8	0	0	0	0	0

[a] Numbers of different characters compared with the profile of *Pseudomonas fuscovaginae* defined for eight biochemical characters (levan production, Kovac's oxidase, arginine dihydrolase, 2-ketogluconate production, and acid production from inositol, sucrose, sorbitol, and trehalose). All strains were gram-negative and produced a water-soluble fluorescent pigment on KB medium.
[b] P+ = Pathogenic in 3-wk-old rice plants of variety IRAT 13, P− = not pathogenic on rice plants, S+ = positive serological reaction with GR2 antiserum (serum diluted to 1/200), and S− = negative serological reaction.

and BM1 (Japan); HMB264 (Burundi); GR2 (Madagascar); and BCE32 and 532 (Colombia) (10).

Biochemical characteristics. The biochemical characteristics of bacterial strains were determined by the microbiological techniques described by Fahy and Hayward (4) and Lelliott and Stead (6). Acid production from sucrose, inositol, sorbitol, and trehalose (1%) was tested on the medium of Ayers et al (4).

Serological technique. An antiserum for strain HMB266 of *P. fuscovaginae* was supplied by H. Maraite, whereas antisera for strain GR2 were prepared in the CIRAD-IRAT (CIRAD = Centre de Coopération Internationale en Recherche Agronomique pour le Développement; IRAT = Institut de Recherches Agronomiques Tropicales et des Cultures Vivriéres) Plant Pathology laboratory in Montpellier (2,10). The antisera were stored at −20 C in glycerol (50%). Bacteria were grown 48 hr on nutrient agar (Difco) at 28 C for the tube agglutination test. For this test, 0.5 ml of antiserum diluted 1/100 with saline was mixed with 0.5 ml of bacterial suspension (10^9 colony-forming units [cfu] per milliliter). After 1 hr, a positive test was indicated by the appearance of a flocculent precipitate, observed with ×10 magnification. Some strains reacted more weakly than others. Nevertheless, this variability was not always stable when the test was repeated and it was not taken into consideration. The agglutination titer of the antiserum for the GR2 strain was 1/800 with strain GR2. The agglutination titer of the antiserum for the strain HMB266 from Burundi (a strain that was not available) was 1/800 against strain HMB264, another strain from that country.

Pathogenicity test. Fifteen rice seedlings were inoculated with the reference strains of *P. fuscovaginae* and 10 plants with the unknown strains (10). Bacterial cultures (24 hr old) grown on KB medium were harvested in sterile distilled water. Aqueous cell suspensions (10^8 cfu/ml) were injected with a syringe into 3-wk-old rice seedlings (variety IRAT 13) between leaf sheaths about 5 cm above soil until inoculum filled up intersheath spaces. Plants were grown in a greenhouse in a medium composed of peat and pozzolana (volcanic sand) (1:5). The photoperiod was natural (14 hr of daylight), and the temperature varied between 19 C at night and 31 C during the day.

RESULTS

The seven known strains of *P. fuscovaginae* were oxidase and arginine dihydrolase positive and produced acid from trehalose. They did not produce 2-ketogluconate, levan on a sucrose-rich medium, or acids from inositol, sucrose, or sorbitol. All of the strains were pathogenic on rice seedlings (Table 1) and could be reisolated from diseased plants. Two to 3 days after plants were inoculated, elongated water-soaked blotches were observed on the leaf sheaths. These blotches quickly developed into a brownish black necrosis that eventually spread the full length of the sheaths. Rice seedlings inoculated with strain GR2 often died within 7–14 days (Table 1). Strain 532 from Colombia was only very slightly pathogenic in our experimental conditions (three of 15 plants had symptoms of sheath necrosis). The pathogenicity of most reference strains was repeated several times and they always reacted alike. Strains 6801, HMB264, and GR2 were agglutinated by antiserum GR2, but strains 7103, BM1, 532, and BCE32 were not.

One hundred and thirty-six fluorescent, gram-negative strains of bacteria were isolated from rice grains or diseased leaf sheaths from five countries: Brazil, 6; Cameroon, 12; Madagascar, 87; Reunion, 26; and Rwanda, 5. Thirty-six of the 136 strains had the same biochemical profile for eight characteristics as the seven reference strains of *P. fuscovaginae* (Table 2). Twenty-four of the 36 strains reacted with the antiserum for the GR2 strain of *P. fuscovaginae* and were pathogenic to rice seedlings (Fig. 1), causing typical elongated water-soaked blotches beginning 2–3 days after inoculation. These blotches developed into a brownish black necrosis similar to that produced by the seven known strains of *P. fuscovaginae*. Plants of variety IRAT

13 were frequently killed by the unknown strains. Ten strains did not react with antiserum to the GR2 strain but were pathogenic to rice seedlings, producing typical symptoms. The remaining two strains did not react with either antisera and were not pathogenic on rice.

Among the 100 strains for which the biochemical profile differed from that of *P. fuscovaginae* by at least one of the eight characteristics, 76 were not pathogenic on rice (Fig. 1). Three of these strains reacted with antiserum GR2 and the remainder did not. None of the 24 pathogenic strains reacted with antiserum GR2. In inoculated plants, brown longitudinal necrosis on leaf sheaths and blades developed after 4–5 days. The necrosis rarely progressed to death of the plants within 3 wk after inoculation. Except by the death of plants, these symptoms could not be differentiated from those induced by *P. fuscovaginae*.

When the eight biochemical tests were used for comparison, some of the unknown strains differed from *P. fuscovaginae* only for production of 2-ketogluconate and acid from trehalose. Other strains differed for production of levan, 2-ketogluconate, and acids from inositol, saccharose, and sorbitol.

All of the reference strains of *P. fuscovaginae*, except for BCE32 and 532 (originating from Colombia), were agglutinated by antiserum HMB266, whereas with antiserum GR2, only strains 6801, HMB264, and GR2 reacted positively (Table 1). Thirty-one selected unknown strains out of the 136 reacted in the same way (Table 3). Among the 22 strains that were pathogenic and had a biochemical profile identical to that of *P. fuscovaginae*, 12 reacted with both antisera, eight reacted positively with the antiserum HMB266 but not with antiserum GR2, and two strains did not react with either antiserum. Two strains with a biochemical profile identical to that of *P. fuscovaginae*, but nonpathogenic on rice, and four pathogenic strains with an atypical biochemical profile did not react with either of the two sera (Table 3). Three strains with a biochemical profile different from that of *P. fuscovaginae* and nonpathogenic on rice reacted positively with each antiserum.

DISCUSSION

Reference strains of *P. fuscovaginae* had identical biochemical profiles and were pathogenic on rice but reacted differently with antisera (10). Bacterial strains that had the same biochemical profile as *P. fuscovaginae* and were pathogenic on rice were considered as belonging to this bacterial species. A positive serological reaction adds information for identification, but a negative reaction was not exclusive.

When the biochemical, serological, and pathogenicity tests were applied to 136 fluorescent gram-negative bacterial strains from rice, 34 strains originating from Madagascar and Rwanda were identified as *P. fuscovaginae*. This supports earlier reports on the presence of *P. fuscovaginae* in Madagascar (3,10) and Rwanda (2). The majority of these strains reacted with at least one of the two antisera used.

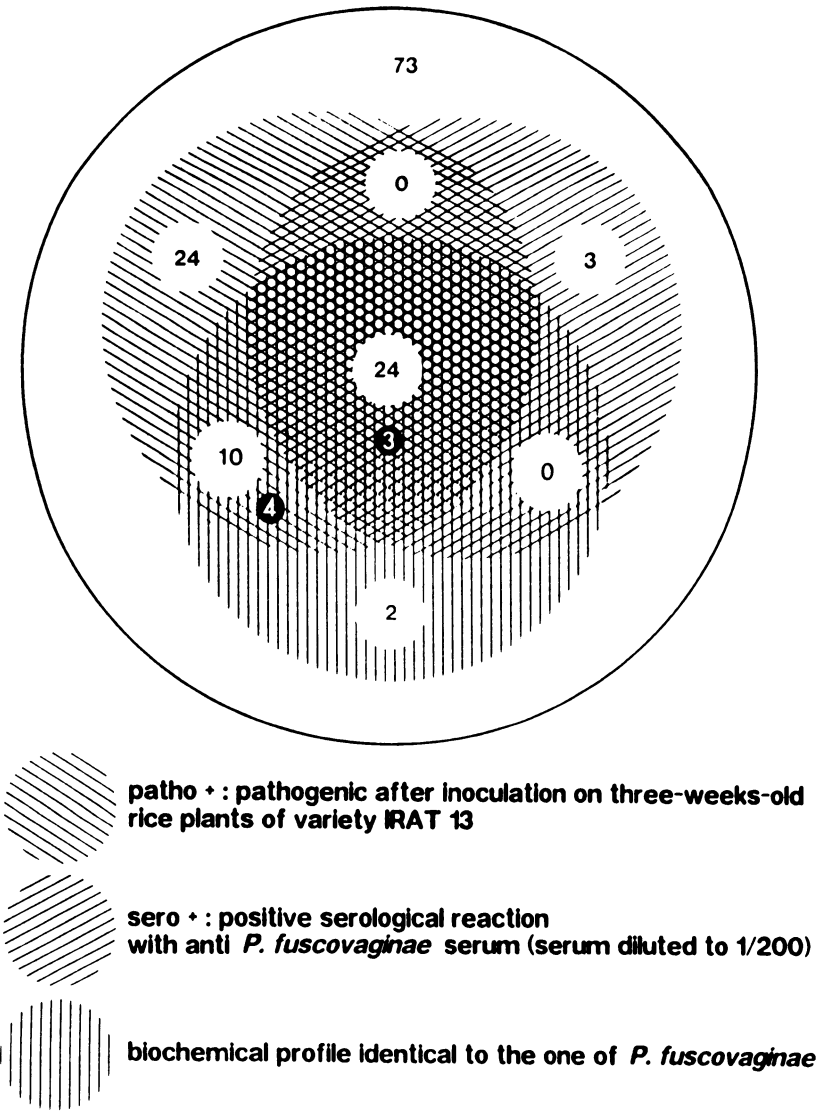

Fig. 1. Combined biochemical, serological, and pathological properties for 136 fluorescent bacterial strains from rice (O) and seven reference strains of *Pseudomonas fuscovaginae* (●).

Table 3. Serological reaction of 31 unidentified bacterial strains of rice

Origin	Number of strains	Biochemical profile[a]	Pathogenicity[b]	Tube agglutination[c] antiserum[d]	
				Strain GR2	Strain HMB266
Madagascar	12	+	+	+	+
Madagascar	7	+	+	−	+
Madagascar	2	+	+	−	−
Madagascar	2	−	+	−	−
Rwanda	1	+	+	−	+
Reunion	2	+	−	−	−
Reunion	2	−	+	−	−
Cameroon	3	−	−	+	+t

[a] + = Biochemical profile identical to *Pseudomonas fuscovaginae* (positive reaction for oxidase, arginine dihydrolase, and production of acids from trehalose; negative reaction for production of 2-ketogluconate, levan, and acids from inositol, sucrose, and sorbitol); − = biochemical profile different from *P. fuscovaginae* by at least one character.

[b] + = Pathogenic bacteria after inoculation on 3-wk-old rice plants of variety IRAT 13; − = bacteria not pathogenic.

[c] + = Flocculent precipitate formation, − = absence of flocculent precipitate, and +t = late positive reaction (1–3 hr).

[d] Diluted to 1/200.

The hypersensitivity test on tobacco proposed by Miyajima et al (8) was not used for identification of *P. fuscovaginae* because variability in results occurred in an earlier study (10). We also considered immunofluorescence for the serological assay, but serological cross-reactions were more frequent than in agglutination tests (P. Rott, *unpublished*), which led us to routinely apply the latter technique.

Biochemical profile, pathogenicity, and serology, when considered separately, did not permit accurate identification of *P. fuscovaginae*. Among the 102 fluorescent gram-negative strains that were different from *P. fuscovaginae*, two strains were found with a biochemical profile identical to that of *P. fuscovaginae* but were not pathogenic and had a negative serological reaction. The possibility that strains had lost pathogenicity was not taken into consideration because the bacteria were tested within a short period after isolation.

Further biochemical characterization permitted us to differentiate these strains from *P. fuscovaginae* but only by a few minor characteristics, such as the use of L-arabinose and galactose (P. Rott, *unpublished*). Therefore, it cannot be completely excluded that these two strains were members of the *P. fuscovaginae*. Some known strains of *P. fuscovaginae* were also only weakly pathogenic in our study. Furthermore, these results could be explained by this organism being an opportunistic weak pathogen that is simply a variant of the commonly encountered epiphytic pseudomonads.

In Madagascar, bacterial sheath brown rot appeared to be widespread in irrigated rice only at elevations of between 1,300 and 2,000 m (3) where low temperatures can predispose the plants to infection by *P. fuscovaginae* (7). In the tests reported here, 24 of the 102 strains were pathogenic to rice but had a different biochemical profile (five or six different characteristics) and a negative serological reaction. They were not considered to be *P. fuscovaginae*, and a more detailed study of these strains will be necessary to identify them and determine their importance in the development of symptoms of necrosis in the field. They did not belong to *P. syringae* because they were arginine dihydrolase and oxidase positive and produced 2-ketogluconate.

Three strains that were positive in the serological test but different biochemically from *P. fuscovaginae* were not pathogenic. Thus, cross-reactions can occur between nonpathogens and two different antisera to *P. fuscovaginae*. These cross-reactions were confirmed by H. Maraite in Belgium (H. Maraite, *personal communication*).

Biochemical profile, pathogenicity, and serology, when considered two by two, permit accurate identification of *P. fuscovaginae*. All strains that were pathogenic and agglutinated by antiserum GR2 had the same biochemical profile of eight characteristics (Fig. 1). Strains that were agglutinated by antiserum GR2 and had the same biochemical profile were pathogenic on rice. Twenty-four of 34 strains that were pathogenic and had the same biochemical profile as *P. fuscovaginae* were agglutinated by antiserum GR2. The remaining 10 belonged to *P. fuscovaginae*, because some known strains of this bacterial species did not react either (10).

A positive reaction for pathogenicity (development of brownish black necrosis of the sheath of rice plants) and serology (agglutination with an antiserum to *P. fuscovaginae*); serology and biochemical profile (agreement with all eight characteristics); or pathogenicity and biochemical profile were required to identify *P. fuscovaginae*. However, only the combination of pathogenicity and biochemical profile permitted identification of all strains of the pathogen. Consequently, this combination of diagnostic tests is recommended for the identification of *P. fuscovaginae* and differentiation from other fluorescent pseudomonads on rice.

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