Diversity of *Pseudomonas fuscovaginae* and Other Fluorescent Pseudomonads Isolated from Diseased Rice

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ABSTRACT

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Fluorescent pseudomonads associated with sheath brown rot of rice were isolated from rice at low and high elevations in various geographic areas and analyzed for pathogenicity and phenotypic and genetic diversity by biochemical tests and restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S-rDNA genes. Standard strains of various *Pseudomonas* species of rRNA homology group I were analyzed along with field-collected isolates. Biochemical characterization revealed 23 groups characterized by different nutritional features. Nineteen of thirty-five rice isolates were identified as *P. fuscovaginae* (biochemical group G1). Using PCR-RFLP, 25 composite 16S-rDNA haplotypes were found. Parsimony analysis revealed that the *P. fuscovaginae* isolates were distributed in four 16S-rDNA haplotypes, but they formed a cluster that differentiated them from other rRNA group I *Pseudomonas* species. All field isolates were pathogenic on rice when introduced by injection. Inoculation of rice without wounding at the booting stage showed that only the four haplotypes of *P. fuscovaginae* and isolates with haplotypes 7, 9, and 17 were pathogenic. Thus, populations of rice-associated fluorescent pseudomonads comprised both phylogenetically different pathogenic isolates and opportunistic isolates pathogenic on rice only after wounding.

Pseudomonas fuscovaginae is a fluorescent pseudomonad that causes sheath brown rot of rice in temperate regions (22). This pathogen has been described in countries where cold temperatures represent a limiting factor for rice cultivation: northern Japan, Nepal, Madagascar, Burundi, and Colombia (5,30,33,36,40). The most important damage associated with the bacterium is grain sterility. Up to 58% loss in yield was recorded in Japan, where 160,000 ha was affected by sheath brown rot in 1976 (21). *P. fuscovaginae* is properly characterized using biochemical tests, but serological characterization appears to be difficult (29,30,41). Although *P. fuscovaginae* is closely associated with sheath rot, other pathogenic fluorescent pseudomonads are responsible for sheath rot symptoms in Madagascar, Burundi, and Colombia (5, 29,41). The genetic relationships between *P. fuscovaginae* and other fluorescent pseudomonads pathogenic to rice remain unknown.

Despite the variety of phenotypic traits encountered among the fluorescent pseudomonads (26), biochemical characterization of biovars has been difficult. However, molecular characterization allows identification and classification of phytopathogenic bacteria at both the genus (3,20) and species levels (15,18), as well as typing at the intraspecies level (1,2,10,17,23). Owing to a low rate of mutation and concerted evolution, the 16S-rRNA gene is probably the target sequence most useful for phylogenetic analysis and typing of bacteria at the species level (15,19,34,38). Recent development of the polymerase chain reaction (PCR) provides a powerful tool for analysis of microorganisms at the species level (13,15,16). A PCR approach based on restriction fragment length polymorphism (RFLP) of 16S-rRNA–encoding sequences has been successfully developed to differentiate species within different genera (12,15,16,27). PCR-RFLP analysis of the 16S-rDNA has been proposed for identification and classification of members of the fluorescent *Pseudomonas* group (16).

Our goals were to characterize and classify fluorescent pseudomonads isolated from sheath rot of rice in various geographic areas. We used biochemical and pathogenicity tests and RFLP analysis of PCR-amplified 16S-rDNA on both field isolates and type or reference strains of various pseudomonads belonging to rRNA homology group I (26).

MATERIALS AND METHODS

Bacterial strains. The *Pseudomonas* strains studied are listed in Table 1. Diseased flag leaf sheaths and grains of rice were randomly collected at five locations: four in the highlands in Madagascar in 1991 and 1992 and one in Nepal in 1992. One field per location and 10 plants per field were sampled. Depending on the plant, one to five fluorescent pseudomonads were isolated at CIRAD (Montpellier, France) from diseased sheaths and grains on modified King's medium B (28). Isolates from the Philippines and Indonesia were isolated from sheath rots at IRRI (Los Baños, the Philippines) and at the Institute of Seed Pathology (Copen-

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hagen), respectively. All isolates were evaluated for pathogenicity. Only one isolate per plant origin showing pathogenicity on rice was kept for further testing. Strains identified as *P. fuscovaginae* were subcultured from type strain NCPPB3085 in Japan, Belgium, and Colombia. The other *Pseudomonas* strains belonging to rRNA homology group I were obtained from established collections (Table 1). *Escherichia coli* DH5α was used as a host strain for cloning. The bacteria were stored in sterile distilled water at –20°C.

Molecular cloning, sequencing, and hybridization. Plasmid pGEM-T (Promega, Madison, WI) was used for cloning and sequencing PCR-amplification products as recommended by the manufacturer. T4 DNA ligase was purchased from GIBCO-BRL (Cergy Pontoise, France). Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim (Meylan, France). Sequencing was performed with Sequenase II (Amersham, Little Chalfont, UK) using dideoxynucleotide chain termination (32) with a double-stranded template. Southern blotting and hybridization at 65°C in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) were conducted as described by Sambrook et al. (31). DNA was labeled using ^{32}P-αdCTP and the Rediprime random primer labeling kit from Amersham.

Biochemical characterization. Isolates were characterized by eight biochemical tests useful in identifying *P. fuscovaginae* (29; Table 2). *P. fuscovaginae* strains have the G1 biochemical pattern.

PCR amplification. Bacteria were grown on Luria-Bertani liquid medium (31) with shaking at 37°C for 24 h. Cells were harvested by centrifugation (12,000 × *g*, 10 min), washed with sterile distilled water, and resuspended in sterile distilled water to give an OD_{600} of 0.5. A 1-µl sample of bacteria was mixed with all PCR reagents, and DNA amplification was conducted on crude bacterial extract after lysis at 95°C for 5 min in a Perkin-Elmer Cetus (Nowalk, CT) thermocycler. The primers used for DNA amplification, fD1 and rD1, corresponded to the 5′ end (5′-AGAGTTTGATCCTGGCTCAG-3′) and 3′ end (5′-TAAGGAGGTGATCCAGGC-3′) of the 16S-RNA gene, respectively (38). PCR reactions were conducted in a final volume of 100 µl with 0.1 µM of each primer, 1.5 mM $MgCl_2$, 20 µM dNTPs, and 2.5 units of

TABLE 1. *Pseudomonas* strains and rice sheath rot isolates

Strain designation[y]	Species and biovar	Origin
CFBP2102^T (ATCC13525)	*P. fluorescens* bv. I	Water, U.K.
LMG2215^T (ATCC10844)	*P. fluorescens* bv. II-1	*Cichorium intibus*, U.S.
LMG2238^T (ATCC13889)	*P. fluorescens* bv. II-2	*Pastinaca sativa*, U.S.
CFBP2127 (ATCC17400)	*P. fluorescens* bv. III	Hen egg, U.S.
CFBP2129 (ATCC17513)	*P. fluorescens* bv. IV	Water by hydrocarbon enrichment, CA
CFBP2131 (ATCC17518)	*P. fluorescens* bv. V	Soil
CFBP2124 (ATCC17552)	*P. fluorescens* bv. VI	Water, U.S.
CIP103295^T (ATCC13985)	*P. aureofaciens*	River clay
CFBP2132^T (ATCC9446)	*P. chlororaphis*	Plate contaminant
CFBP2068^T (ATCC33618)	*P. tolaasii*	*Agaricus bisporus*, U.K.
CFBP2107^T (ATCC13223)	*P. viridiflava*	*Phaseolus* sp., Switzerland
CFBP1392^T (ATCC19310)	*P. syringae* pv. *syringae*	*Syringa vulgaris*, U.K.
LMG2257^T (ATCC12633)	*P. putida* bv. A	Soil, U.S.
ATCC17472	*P. putida* bv. B	Soil by tryptophan enrichment, CA
ATCC17484	*P. putida* bv. B	Soil by naphthalene enrichment, U.K.
CFBP2130 (ATCC17386)	*P. putida* bv. C	Water by tryptophan enrichment, CA
CFBP2101^T (ATCC10857)	*P. cichorii*	*Cichorium endivum*, Germany
CFBP1477	*P. cichorii*	*Lactuca scariola*, France
CFBP2443^T (ATCC17588)	*P. stutzeri*	Spinal fluid
LMG2158^T (NCPPB3085)	*P. fuscovaginae*	*Oryza sativa*, Japan
BCE3^T (NCPPB3085)	*P. fuscovaginae*	*O. sativa*, Japan
6801^T (NCPPB3085)	*P. fuscovaginae*	*O. sativa*, Japan
V3, V4.2, V6′, V7.1, V10, V10′, Vgr11′.3, Sbi7, Sbpg2, F2.1, F2.2, F3, F4, F5, F7.3, Fgr9.1	Unknown	*O. sativa*, Madagascar (1991)[z]
V1A, V3A, V4A, V6A, V9A, S4A2, S4B, Sd1.1, Sd7.2, Sd8.1	Unknown	*O. sativa*, Madagascar (1992)[z]
Lum3, Lum5.2, Lum6.1, Lum6.2, Lum10	Unknown	*O. sativa*, Nepal (1992)[z]
DIGSP31.713	Unknown	*O. sativa*, Indonesia
5200, 6031	Unknown	*O. sativa*, the Philippines

[y] Reference *Pseudomonas* strains were obtained from the French Collection of Phytopathogenic Bacteria (CFBP), Angers; the Collection of the Pasteur Institute (CIP), Paris; the Laboratory of Microbiology, Rijskuniversteit of Gent (LMG), Belgium; and the American Type Culture Collection (ATCC), Rockville, MD. Alternate strain number from the ATCC or the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, UK, is given in parentheses. T = type strain.

[z] Year of sheath rot collection.

TABLE 2. Biochemical patterns of reference rRNA group I pseudomonads and rice sheath rot isolates

Characteristic	Biochemical pattern[y]																						
	G1[z]	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23
Kovac's oxidase	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	–	–	+	+	+	+	+
Levan production	–	–	–	–	+	+	+	–	–	–	–	+	+	–	+	–	–	+	–	–	–	–	–
2-Ketogluconate production	–	–	+	–	+	+	+	–	+	–	–	–	+	+	–	+	+	–	+	–	–	–	+
Arginine dihydrolase	+	+	+	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–	+	+	–	–	+
Acid production from:																							
Sucrose	–	+	–	–	+	+	+	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–
Sorbitol	–	+	+	–	+	–	+	–	+	–	–	–	+	–	+	–	+	+	–	–	–	+	–
Trehalose	+	+	+	–	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–
Inositol	–	+	+	–	–	+	+	+	–	–	–	–	+	+	+	–	+	+	–	–	+	+	–

[y] + = positive reaction; – = negative reaction.

[z] Biochemical pattern of *P. fuscovaginae*.

Taq DNA polymerase (Promega) in the buffer supplied by the manufacturer. DNA was amplified over 35 cycles of denaturation for 1 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 2 min. After the last cycle, DNA was extended at 72°C for 3 min. Amplification was confirmed by analyzing 5 µl of each PCR reaction mixture on a 0.9% agarose minigel (31).

RFLP analysis of PCR products. PCR products were digested with each of nine restriction endonucleases, *Alu*I, *Rsa*I, *Hae*III, *Hin*fI, *Cfo*I, *Nde*II, *Msp*I, *Dde*I, and *Taq*I, according to the supplier's (GIBCO-BRL) recommendations; 8 µl of the PCR products was incubated with 10 units of enzyme. Digestion products were separated on 3% Nusieve agarose gels (FMC, Rockland, ME) in 1× Tris-acetate EDTA buffer (31) at 6 V/cm for 1 h. Gels were stained with ethidium bromide and photographed with UV light.

From the combination of the restriction patterns obtained with the nine restriction endonucleases, each strain was assigned a composite 16S-rDNA haplotype. The 16S-rDNA sequences of *P. aeruginosa* strain DSM50071 (GenBank accession M34133), *P. mendocina* strain ATCC25411 (GenBank accession M59154), and *P. putida* strain PaW1 (GenBank accession L28676) were used to estimate the map locations of the restriction sites in the 16S-rDNA genes. Differences in restriction sites between the composite 16S-rDNA haplotypes were analyzed by the parsimony method by the computer program PAUP (phylogenetic analysis using parsimony) (35).

TABLE 3. Distribution of reference rRNA group I pseudomonads and rice sheath rot isolates within the 16S-rDNA haplotypes and biochemical groups

Strain	16S-rDNA haplotype[x]	Biochemical group[y]
CFBP2102 (*P. fluorescens* bv. I)	1	G13
LMG2215 (*P. fluorescens* bv. II-1)	1	G13
CFBP2127 (*P. fluorescens* bv. III)	1	G14
LMG2238 (*P. fluorescens* bv. II-2)	2	G13
CFBP2131 (*P. fluorescens* bv. V)	3	G3
V4A, Sd1.1, S4B, F7.3, DIGSP31.713, 5200	3	G7
Fgr9.1	3	G6
V10′	3	G3
F2.1	3	G5
CFBP2132 (*P. chlororaphis*)	4	G6
CIP103295 (*P. aureofaciens*)	5	G6
CFBP2068 (*P. tolaasii*)	6	G3
Lum10	7	G10
CFBP2107 (*P. viridiflava*)	8	G17
CFBP1392 (*P. syringae* pv. *syringae*)	9	G18
CFBP2129 (*P. fluorescens* bv. IV)	9	G15
V1A	9	G11
V3A	9	G12
ATCC17472 (*P. putida* bv. B)	10	G20
LMG2257 (*P. putida* bv. A)	11	G19
PaW1 (*P. putida*)[z]	11	Not determined
Sd8.1, Sd7.2, V3, V6′, V7.1, V10A, Sbpg2, F3	12	G1
LMG2158, BCE3 (*P. fuscovaginae*)	13	G1
Sbi7, S4A2, F4, F5, V4.2, V6A, V9A, Lum3, Lum5.2, Lum6.1	13	G1
F2.2	14	G1
6801 (*P. fuscovaginae*)	15	G1
Vgr11′.3	16	G4
6031	17	G9
ATCC17484 (*P. putida* bv. B)	18	G20
CFBP2124 (*P. fluorescens* bv. VI)	18	G16
CFBP2101 (*P. cichorii*)	19	G21
CFBP1477 (*P. cichorii*)	20	G22
CFBP2443 (*P. stutzeri*)	21	G23
CFBP2130 (*P. putida* bv. C)	22	G8
Sd4	23	G2
Lum6.2	23	G8
ATCC25411 (*P. mendocina*)[z]	24	Not determined
DSM50071 (*P. aeruginosa*)[z]	25	Not determined

[x] Haplotypes are defined in Tables 4 and 5.

[y] Biochemical groups are defined in Table 2.

[z] For these strains, 16S-rDNA haplotypes were determined from the 16S-rDNA sequences available in GenBank; PaW1: GenBank accession L28676, ATCC25411: GenBank accession M59154; DSM50071: GenBank accession M34133.

Pathogenicity. Bacteria were grown on plates on King's medium B (14) for 24 h at 28°C. Bacteria were resuspended in sterile distilled water and adjusted to an OD_{530} of 0.6 (10^9 CFU/ml). Each strain tested was inoculated to the cultivar Dourado Precoce, which is susceptible to *P. fuscovaginae*, at two plant growth stages. First, 15 plants were inoculated at the three- to four-leaf stage by injecting 100 µl of bacterial suspension at 10^8 CFU/ml between leaf sheaths, using a hypodermic syringe. The number of plants with sheath necrosis was recorded after 7 days. Five plants were inoculated at the booting stage by pouring 100 µl of bacterial suspensions (10^8 or 10^4 CFU/ml) on the adaxial side of the flag leaf sheath. After 7 days, the number of plants with sheath rot symptoms was recorded, and the lesion length was measured. For both inoculation methods, control plants were inoculated with sterile distilled water. Plants were grown in a greenhouse on horticultural compost. Twenty-four hours before inoculation, plants were transferred to a growth chamber at 18°C, 80% relative humidity, and 12 h of light, where they were kept for 7 days after inoculation.

RESULTS

Biochemical characterization of *Pseudomonas* isolates from rice and reference pseudomonads. Analysis of the distribution

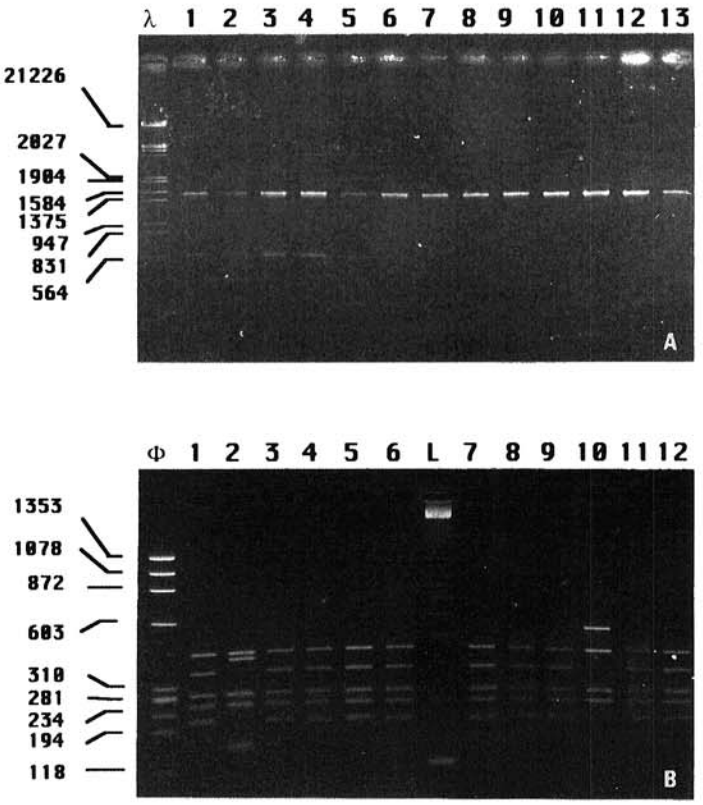

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified 16S-rDNAs and restriction digestion products for selected *Pseudomonas* strains and enzymes. **A,** PCR products amplified with 16S-rDNA–specific primers. Lane λ: lambda DNA digested by *Hin*dIII and *Eco*RI; sizes are given in base pairs. Lanes 1–5: *P. fuscovaginae* strains Sd8.1, Lum3, BCE3, LMG2158, and 6801, respectively. Lanes 6–13: *Pseudomonas* spp. LMG2238, LMG2215, LMG2257, V4A, Vgr11′.3, V1A, V3A, and F7.3, respectively. **B,** *Cfo*I restriction fragments of amplified 16S-rDNAs. Φ: Φ× 174 DNA digested by *Hae*III; sizes are given in base pairs. Lanes 1–6: *Pseudomonas* spp. V3A, Lum6.2, V4A, Sd1.1, V1A, and 5200, respectively. Lane L: 123-pb ladder. Lanes 7–12: *Pseudomonas* spp. 31.713, S4B, F7.3, Lum10, 6031, and Fgr9.1, respectively.

of the 8 biochemical characteristics previously described by Rott et al. (29) revealed 12 patterns, from G1 to G12, among the 35 rice isolates of *Pseudomonas* spp. (Table 2). The group G1, which is characteristic of *P. fuscovaginae*, contained 19 of 35 isolates tested and the type strain of *P. fuscovaginae* obtained from three different collections (Table 3). Of the remaining 16 isolates, 6 were in group G7, and the other biochemical patterns were represented by individual strains.

Biochemical analysis revealed that 14 of the 19 reference rRNA group I pseudomonads were distributed in 11 patterns, from G13 to G23 (Table 2), distinct from those of rice isolates. Strains of *P. fluorescens* bvs. I, II-1, and II-2 showed the same pattern and constituted group G13, and the two strains of *P. putida* bv. B (ATCC17484 and ATCC17472) were sorted into group G20 (Table 3). However, the remaining 5 reference strains shared the same biochemical pattern as rice isolates, with the strains of *P. fluorescens* bv. V and *P. tolaasii* in group G3, the strains of *P. chlororaphis* and *P. aureofaciens* in group G6, and the strain of *P. putida* bv. C in group G8.

Amplification of the 16S-RNA–encoding sequence. Amplification of the 16S-rRNA gene for each strain yielded the expected approximately 1.5-kb PCR product corresponding to the full-length gene. In addition, a 0.5-kb fragment was consistently found in samples of *P. fuscovaginae* that was never found in other *Pseudomonas* species (Fig. 1A, lanes 1–5).

To determine if this 0.5-kb fragment was related to the 16S-rDNA sequence, PCR products from *P. fuscovaginae* isolates as well as other isolates were transferred onto nylon membrane and hybridized with a 16S-23S rDNA probe from *E. coli* (11). The 1.5-kb product corresponding to the 16S-rDNA was recognized by the probe in each sample, whereas the 0.5-kb amplification product found in *P. fuscovaginae* did not hybridize (data not shown). PCR products from strain V10A of *P. fuscovaginae* were cloned into pGEM-T. Several clones bearing the 0.5-kb fragment were selected, and the insert was sequenced on both strands. Computer analysis showed the presence of a putative open reading frame on each strand, but sequence comparison revealed no homology with any sequence present in the GenBank database using the DNASTAR program, version 1.59.

To investigate whether the 0.5-kb fragment was specific to *P. fuscovaginae*, a PCR assay was conducted using two primers determined from the sequence of the 0.5-kb insert: fus1 (5′-GATCAGGTAGGGCGAACAGA-3′) and fus2 (5′-CGACGAAAAGGCCCTGATCA-3′). The following strains were tested: *P. putida* bv. A LMG2257; *P. fluorescens* bvs. II.1 LMG2215, IV CFBP2129, and V CFBP2131; *P. syringae* strain CFBP1392; and *P. fuscovaginae* isolates LMG2158, Sd8.1, 6801, and F2.2. The *P. fuscovaginae* isolates yielded an approximately 0.5-kb band, whereas other *Pseudomonas* species yielded several bands ranging from 1 to 0.4 kb (data not shown). When southern blots of the PCR products were probed with the PCR product from *P. fuscovaginae* isolate Sd8.1, a 0.5-kb hybridizing band was detected in all the strains tested, which indicated that the 0.5-kb fragment was not specific to *P. fuscovaginae*.

Characterization of rice isolates by RFLP analysis of 16S-rDNA genes. The PCR products generated with the primer pair fD1/rD1 were digested with each of nine restriction enzymes. Since the presence of the additional 0.5-kb fragment in *P. fuscovaginae* samples generated restriction fragments that could be confused with fragments from the 16S-rDNAs, 0.5-kb PCR products from several *P. fuscovaginae* isolates were cloned, amplified using the primer pair fus1/fus2, and digested with the same nine restriction enzymes. The comparison of the total PCR products to the 0.5-kb–specific bands differentiated the bands generated by the digestion of the 0.5-kb fragment from whole-cell PCR products.

Restriction of the 16S-rDNAs yielded between three and seven bands depending on the enzyme and the strain (Fig. 1B). Three to six different restriction pattern types were recorded depending on the enzyme (Table 4). The map locations of the restriction sites in the 16S-rDNA genes were estimated by comparison to the known rDNA sequences of *P. aeruginosa* and *P. mendocina* (16) and *P. putida*. Among the 77 restriction sites analyzed, representing 304 bp, 35 were polymorphic within the sample of *Pseudomonas* strains (Table 5). The combination of the different data from the nine

TABLE 4. Composite 16S-rDNA pseudomonad haplotypes determined by restriction analysis of polymerase chain reaction (PCR)-amplified products

16S-rDNA haplotype[y]	No. of strains	Similar restriction patterns of amplified 16S-rDNA genes digested with enzymes[z]								
		*Alu*I	*Rsa*I	*Hae*III	*Hin*fI	*Cfo*I	*Nde*II	*Msp*I	*Dde*I	*Taq*I
1	3	A	A	A	A	A	A	A	A	A
2	1	A	A	B	A	A	A	A	A	A
3	10	A	A	A	A	A	A	A	B	A
4	1	C	C	A	A	A	A	A	A	A
5	1	B	C	A	A	A	A	A	A	A
6	1	A	A	A	A	A	A	B	B	A
7	1	D	A	C	E	C	A	B	D	A
8	1	D	A	A	C	A	A	A	B	A
9	3	C	B	A	A	A	A	A	A	A
10	1	B	A	A	E	A	A	A	B	A
11	2	D	D	A	D	A	A	A	B	B
12	8	D	A	A	F	A	A	A	A	B
13	12	D	A	A	F	A	D	A	A	B
14	1	D	A	A	F	A	A	A	E	B
15	1	D	A	A	F	A	D	F	A	B
16	1	D	A	A	D	A	A	A	B	B
17	2	D	A	A	B	A	A	A	B	B
18	1	B	A	A	B	A	A	A	A	B
19	1	E	B	A	E	A	A	D	D	C
20	1	E	B	A	E	A	A	D	D	A
21	1	D	A	C	A	C	C	A	A	D
22	1	C	B	C	B	B	B	C	A	A
23	2	C	B	C	A	B	B	B	A	A
24	1	D	E	A	E	A	E	A	F	A
25	1	D	D	D	B	B	C	B	C	E

[y] Haplotypes numbered from 1 to 25 represent the combinations of restriction patterns obtained when PCR-amplified 16S-rDNA was digested with nine individual restriction enzymes. Haplotypes 24 and 25 were defined on the basis of the predicted restriction pattern of *P. mendocina* strain ATCC25411 and *P. aeruginosa* strain DSM50071, respectively, from their known 16S-rDNA sequence.

[z] Groups of similar restriction patterns for each restriction endonucleases are designated by the same uppercase letter (A–F).

restriction enzymes defined 25 composite 16S-rDNA haplotypes (Tables 4 and 5). *P. fuscovaginae* isolates were grouped into 16S-rDNA haplotypes 12 to 15 (Table 3), which showed distinctive restriction patterns with four enzymes: *Hin*fI (group F), *Nde*II (group D), *Msp*I (group F), and *Dde*I (group E). *Hin*fI restriction pattern F differed from *Hin*fI pattern B by the presence of one restriction site, *Hin*fI 935, which was found only in *P. fuscovaginae* isolates (Table 5). Likewise, *Nde*II restriction pattern D differed from *Nde*II pattern A by the presence of restriction site *Nde*II 1005.

The genetic relationships between the 16S-rDNA haplotypes were estimated from the data matrix shown in Table 5 using Dollo's parsimony analysis (35). Twelve equally parsimonious trees were obtained from a heuristic search. These trees showed few differences in topology; the tree resulting from the bootstrap analysis is presented in Figure 2. The four 16S-rDNA haplotypes, including the reference strains and rice isolates identified as *P. fuscovaginae*, formed a clearly delineated cluster. Within this group, the relative distances between pairs of haplotypes varied from 1.3 to 3.9% base substitutions in the restriction sites with an arithmetic mean of 2.2%, which corresponded to one to three differences in restriction sites.

The closest known relative to *P. fuscovaginae* strains was *P. putida* bv. B strain ATCC17484 characterized by 16S-rDNA haplotype 18, which differed from *P. fuscovaginae* haplotypes by two to four restriction sites. These strains were clustered with *P. putida* bv. A type strain and two rice isolates, Vgr11′.3 and 6031, with a mean divergence of 3.9% base substitutions. Isolates Vgr11′.3 (haplotype 16) and 6031 (haplotype 17) differed by only one restriction site and were closely related to the strain of *P. putida* bv. A, with one and two differences in restriction sites, respectively. Among the other rice sheath rot isolates that did not share their 16S-rDNA haplotypes with reference pseudomonads, strain Lum10 (characterized by 16S-rDNA haplotype 7) differed from reference strains by at least six restriction sites (7.9% base substitutions). Finally, strains Sd4 and Lum6.2 (16S-rDNA haplotype 23) differed from *P. putida* bv. C in only two restriction sites.

Pathogenicity of fluorescent *Pseudomonas* isolates to rice. Injection of bacteria between leaf sheaths of rice seedlings caused elongated brown necrosis of sheaths with all the rice isolates, indicating that these isolates were pathogenic to rice at that stage (data not shown); reference strains were not used in this analysis. Bioassays conducted on rice plants at the booting stage showed that the isolates of *P. fuscovaginae* tested were pathogenic at the two concentrations used, 10^4 and 10^8 CFU/ml (Table 6). These isolates caused elongated lesions with brownish-black margins and gray, dry centers. These symptoms were identical to those observed on flag leaf sheaths in fields in Madagascar and Nepal. Both *P. fuscovaginae* strains subcultured from the reference strain NCPPB3085 caused smaller lesions compared with those caused by strains from Madagascar. Among the other rice isolates, the pathogenic isolates caused the same type of symptom as *P. fuscovaginae* isolates. Isolates characterized as belonging to haplotypes 7, 9, and 17 were pathogenic to rice at both concentrations, but isolates Lum10 (haplotype 7), and 6031 (haplotype 17) caused smaller lesions than those caused by *P. fuscovaginae* isolates from Madagascar. Isolates V4A, S4B, and Sd1.1, which belonged to haplotype 3, were weakly pathogenic at 10^8 CFU/ml. All other isolates tested (haplotypes 3, 16, and 23) were not pathogenic to rice at the booting stage. The reference strains tested (*P. fluorescens* bvs. I, II-1, II-2, and V; *P. syringae* pv. *syringae*; and *P. putida* bvs. A and C) were not pathogenic to rice at the booting stage.

TABLE 5. Data matrix of polymorphic restriction sites between 16S-rDNA haplotypes of rRNA group I pseudomonads and rice sheath rot isolates revealed by restriction fragment analysis

Restriction site[y]	16S-rDNA haplotype																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
*Taq*I 65	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
*Alu*I 79	+	+	+	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+
*Hin*fI 90	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	+	−	−	+
*Msp*I 150	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
*Taq*I 151	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
*Cfo*I 162	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
*Rsa*I 174	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
*Dde*I 180	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
*Nde*II 197	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+
*Hae*III 207	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−
*Cfo*I 214	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−
*Hae*III 260	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
*Dde*I 316	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
*Msp*I 379	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
*Rsa*I 428	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
*Dde*I 640	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
*Alu*I 644	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Rsa*I 651	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
*Hae*III 675	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
*Hin*fI 675 (or 490)[z]	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
*Taq*I 740 (or 135)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
*Rsa*I 790 (or 85)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−
*Taq*I 816	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
*Hin*fI 830	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	+	−	−	+	+	−	−	−	+	−
*Nde*II 830	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+
*Nde*II 839	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+
*Alu*I 840	−	−	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−
*Dde*I 843	+	+	−	+	+	−	−	−	+	−	−	+	+	+	+	−	−	+	−	−	+	+	+	+	+
*Taq*I 869	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
*Hae*III 889	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+
*Hin*fI 935 (or 735)	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
*Dde*I 993	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
*Nde*II 1005 (or 665)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
*Dde*I 1031	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+
*Msp*I 1377	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+

[y] The position of each polymorphic restriction site is given according to the nucleotide numbering of the sequence of *P. aeruginosa* strain DMS50071. + and − indicate the presence or absence of the restriction site, respectively.

[z] The position of the restriction site missing in the known sequences was approximated.

DISCUSSION

Biochemical tests and RFLP analysis of PCR-amplified 16S-rDNA were used to characterize fluorescent pseudomonads isolated from rice sheath rot. The results demonstrated that several fluorescent *Pseudomonas* species were associated with rice sheath rot. On the basis of biochemical characterization and 16S-rDNA analysis, a group of fluorescent pseudomonads was identified as *P. fuscovaginae* and could be discriminated from all other isolates. This *P. fuscovaginae* genetic cluster should be added to the different groups of phytopathogenic fluorescent *Pseudomonas* spp. already identified (39). The discrimination of *P. fuscovaginae* from other *Pseudomonas* species was contrary to previous conclusions, based on biochemical analyses, that suggested *P. fuscovaginae* was part of the *P. fluorescens* bv. V group (30). Differing by at least six restriction sites in their 16S-rDNA genes, *P. fuscovaginae* and *P. fluorescens* bv. V formed clearly distinct taxa. *P. fuscovaginae* appeared to be more closely related to the *P. putida* group than to *P. fluorescens,* as defined by the type strains of these two species.

The difference between *P. fuscovaginae* and other fluorescent *Pseudomonas* species was emphasized by the presence of a *P. fuscovaginae*-specific 0.5-kb PCR product when using primers designed for amplification of 16S-rDNA. Although detected only in samples of *P. fuscovaginae* through amplification, this DNA

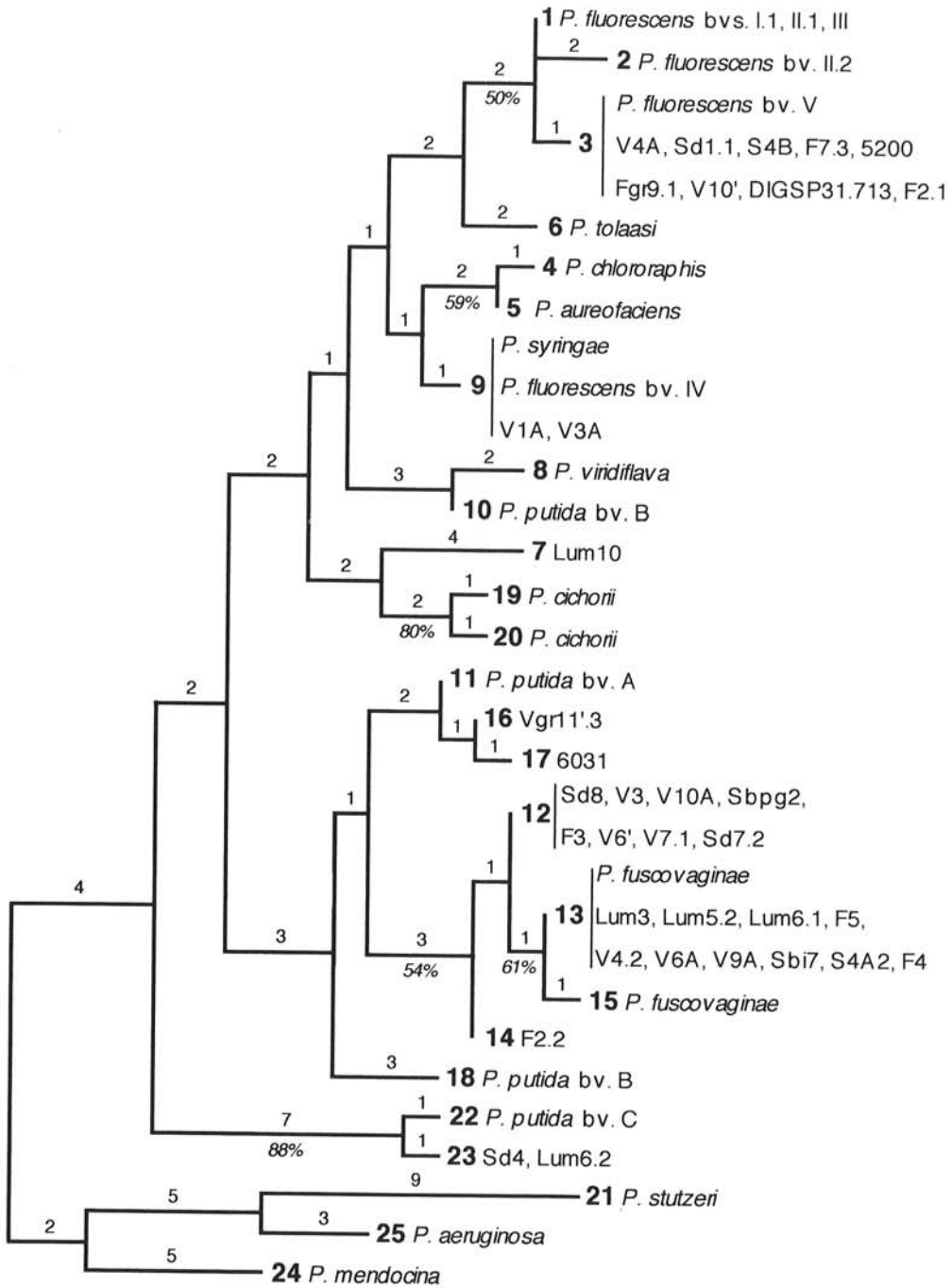

Fig. 2. Phylogenetic tree constructed by the PAUP (35) computer program showing the relationships between the fluorescent pseudomonads isolated from diseased rice and the reference strains of the rRNA group I pseudomonads presented in Table 1. The percentages below the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Boostrap values below 50% are not displayed. The horizontal branches are drawn proportionally to the number of restriction site changes. The numbers above the branches are the total number of changes assigned to each branch by PAUP. The tree length was 89 steps.

sequence was present in all *Pseudomonas* spp. strains studied, as shown by hybridization of PCR products, suggesting that the difference was related to the sequences recognized by the 16S-rDNA primers.

The characterization of *P. fuscovaginae* as a single, homogenous group was based on biochemical characterization, but some genetic diversity was found within *P. fuscovaginae*. Comparison of the groupings obtained by biochemical characterization and genotypic analysis showed that the biochemical G1 group that defined *P. fuscovaginae* strains corresponded to four 16S-rDNA haplotypes (Table 3). Two haplotypes, 12 and 13, included most *P. fuscovaginae* isolates, with 8 and 10 isolates, respectively, whereas haplotype 14 was represented by only 1 isolate. *P. fuscovaginae* reference strain 6801 was the only example of 16-rDNA haplotype 15. This strain, which came from Japan, is a subculture of the type strain NCPPB3085. Two other subcultures of the same type strain, BCE3 from Colombia and LMG2158 from Belgium, were analyzed along with 6801 in the present work and belonged to 16S-rDNA haplotype 13, differing from haplotype 15 in one restriction site. These differences together with serological and morphological variations, previously reported (30), among subcultures of type strain NCPPB3085 suggest that this type strain is a mixture of two isolates of *P. fuscovaginae*. Intraspecies polymorphism has been reported in 16S-rDNA sequences (7). This genetic analysis shows that the *P. fuscovaginae* group is as polymorphic as the cluster constituted by *P. fluorescens* bvs. I, II, III, and V, which are recognized as different subgroups. Therefore, *P. fuscovaginae* isolates might form a cluster of closely related subgroups.

In contrast to *P. fuscovaginae*, the other fluorescent *Pseudomonas* spp. isolates and the reference strains of the rRNA group I *Pseudomonas* species were biochemically and genotypically diverse, because they were characterized by 22 biochemical patterns and 19 16S-rDNA haplotypes (Table 3). However clusterings of reference strains and rice isolates resulting from biochemical or genotypical characterization were not related, since strains with the same 16S-rDNA haplotype could display different biochemical patterns and vice versa. This lack of correlation could be ascribed to the small number of biochemical tests used, which were chosen to identify strains belonging to *P. fuscovaginae*. Our results confirm the usefulness of these tests for this purpose.

The fluorescent pseudomonads isolated from sheath rot of rice were clustered in two groups according to pathogenicity without wounding at the rice booting stage. The strains that were not pathogenic at this stage might be opportunistic pathogens rather than true pathogenic bacteria. Most of these strains, which were isolated either from low or high elevations in different countries, corresponded to 16S-rDNA haplotype 3, which included the *P. fluorescens* bv. V reference strain. The strains that were pathogenic to rice without wounding at the booting stage could be considered true pathogenic species because inoculation in a growth chamber reproduced the field symptoms. They belonged to eight haplotypes. True pathogenic strains from the highlands of Madagascar and Nepal belonged mainly to the *P. fuscovaginae* biochemical and 16S-rDNA groups. Other isolates from Madagascar were closely related to *P. syringae* pv. *syringae*, according to oxidase test and 16S-rDNA analysis, whereas strain Lum10 from Nepal with 16S-rDNA haplotype 7 was not closely related to any type strain. Among the strains from low elevations, isolate 6031 from the Philippines, which was closely related to *P. putida* bv. A, also could be considered a true pathogen. These findings corroborate recent studies in Indonesia (24) and the Philippines (37) showing the occurrence of fluorescent pseudomonads causing sheath rot. Our results show that true pathogenic species are widely distributed in the phylogenetic group of rRNA group I pseudomonads and partly contradict previous studies describing *P. syringae* pv. *syringae* and *P. fuscovaginae* as the only fluorescent *Pseudomonas* species causing rice sheath rot (25,41).

Our results provide information on the structure of the population of fluorescent pseudomonads associated with diseased rice. The genetic diversity found in the *P. fuscovaginae* group should be investigated more thoroughly. Indeed, *P. fuscovaginae* shows a

TABLE 6. Pathogenicity of reference strains of rRNA group I pseudomonads and rice sheath rot isolates on rice inoculated at the booting stage

16S-rDNA haplotype	Strain[w]	No. of diseased sheaths[x]		Mean lesion length[y]	
		10^4 CFU/ml	10^8 CFU/ml	10^4 CFU/ml	10^8 CFU/ml
1	CFBP2102 (*P. fluorescens* bv. I)	0[z]	0		
	LMG2215 (*P. fluorescens* bv. II-1)	0	0		
2	CFBP2238 (*P. fluorescens* bv. II-2)	0	0		
3	CFBP2131 (*P. fluorescens* bv. V)	0	0		
	V4A	0	3		0.7[J]
	S4B	0	4		2.2[H]
	Sd1.1	0	4		1.5[I]
	DIGSP31.713	0	0		
	Fgr9.1	0	0		
	F2.1	0	0		
7	Lum10	4	4	2.0[F]	1.5[I]
9	CFBP1392 (*P. syringae* pv. *syringae*)	0	0		
	V1A	5	5	9.6[A,B]	15.2[C]
	V3A	3	5	7.0[D]	9.8[D]
11	LMG2257 (*P. putida* bv. A)	0	0		
12	Sd7.2	3	5	8.5[C]	15.9[B]
	Sd8.1	4	5	9.0[B]	16.6[A]
13	LMG2158 (*P. fuscovaginae*)	1	5	1.0[G]	9.2[E]
14	F2.2	5	5	10.2[A]	16.8[A]
15	6801 (*P. fuscovaginae*)	5	5	1.6[F,G]	3.2[G]
16	Vgr11′.3	0	0		
17	6031	2	5	6.5[E]	6.4[F]
22	CFBP2130 (*P. putida* bv. C)	0	0		
23	Sd4	0	0		
	Lum6.2	0	0		

[w] Isolates and reference strains tested were selected to be representative of 16S-rDNA haplotypes and biochemical groups. Control plants inoculated with sterile distilled water did not exhibit any symptoms.

[x] Number of diseased sheaths for five inoculated sheaths at the given inoculum concentration.

[y] Means with the same uppercase letter were not significantly different at the $P = 0.05$ level according to Newman-Keuls analysis at the given inoculum level.

[z] Number of replicates varied according to the number of diseased sheaths.

wide host range and has been isolated from sheath rot of maize and sorghum in Burundi, from wheat in Mexico, and from rot of garlic in Réunion Island, France (4,6,9). Studies on other phytopathogenic bacterial species have shown a relationship between population structures and host range and aggressiveness in *Xanthomonas campestris* pv. *citri* (8,10) and geographic origin in *P. solanacearum* (2). Further studies of phylogenetic relationships and pathogenicity within a larger sample of *P. fuscovaginae* isolates are needed to determine whether population structures are related to host range and geographic origin. It also will be interesting to compare more isolates from tropical areas of high and low elevations to evaluate both the geographic distribution and the epidemiological impact of fluorescent *Pseudomonas* species on rice crops.

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