A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov.

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The taxonomic position of a yellow-pigmented group of bacteria, isolated from the phyllosphere of grasses was investigated. Results obtained from restriction analysis of amplified 16S rDNA with seven endonucleases (*CfoI*, *HaeIII*, *AluI*, *HinfI*, *MspI*, *Sau3A* and *ScrFI*) showed identical restriction patterns for each enzyme of all isolates studied, which suggests that all strains belong to the same species. The grass isolates displayed the characteristics of the genus *Pseudomonas*. They were Gram-negative, aerobic and rod-shaped with polar flagella. Isolates were catalase-positive and oxidase-negative, and unable to oxidize or ferment glucose with the production of acid. The isolates did not reduce nitrate to nitrite but were able to utilize a wide range of compounds individually as a sole carbon source, with preference being given to the utilization of monosaccharides. The disaccharides tested were not utilized as substrates. The DNA base compositions of the tested strains ranged from 60 to 61 mol% G+C. The major isoprenoid quinone of each was ubiquinone Q-9 and hydroxy fatty acids were represented by 3-hydroxydodecanoic acid and 2-hydroxydodecanoic acid. Comparison of 16S rDNA sequences showed that the bacteria were members of the genus *Pseudomonas*, with similarity values between 91.5 and 97.7%. DNA–DNA hybridization studies with closely related neighbours revealed a low level of homology (< 27%), indicating that the isolates represent an individual species. On the basis of phenotypic and phylogenetic analyses a new species, *Pseudomonas graminis* sp. nov. (type strain DSM 11363^T), is proposed.

Keywords: *Pseudomonas graminis* sp. nov., plant-associated, phenotypic and phylogenetic analysis

INTRODUCTION

Characterization of community members are a requisite step for ecological studies of bacterial populations in the phyllosphere of plants. Frequently, identification to the level of species is difficult due to the paucity of information on those phyllosphere bacteria that are neither phytopathogenic nor related to bacteria of medical interest (Hirano & Upper, 1992). Thus, recent ecological studies of microbial populations associated with various plants based on phenotypic features revealed groups of bacteria that were not

affiliated to known species (Ercolani, 1991; Legard *et al.*, 1994; Thompson *et al.*, 1993).

Investigations of bacterial community structure on grasses posed the same problems (Behrendt *et al.*, 1997). An unknown group of yellow-pigmented strains was isolated during the whole vegetative period constituting an integral part of the Gram-negative population, with 10^6 – 10^8 c.f.u. (g fresh material)⁻¹. Yellow-pigmented strains are common in the phyllosphere of plants and belong mainly to the genera *Pantoea*, *Xanthomonas*, *Sphingomonas* and *Pseudomonas* (Rudolph *et al.*, 1990). On the basis of conventional physiological and morphological tests, it was not possible in those studies to assign the isolated group of strains to a genus or species. Phenotypic properties

The EMBL accession number for the 16S rDNA sequence from *Pseudomonas graminis* DSM 11363^T reported in this paper is Y11150.

suggested that they were members of the genus *Pseudomonas*, but affiliation was not certain since the description of the genus *Pseudomonas* is too broad (Holt *et al.*, 1994; Palleroni, 1991a).

The present study was undertaken to clarify the taxonomic position of this grass-associated bacterial group. Nineteen strains were selected randomly and extensively characterized by morphological, physiological and chemotaxonomic tests. Analysis of 16S rDNA sequences and DNA–DNA hybridization studies were also performed to study phylogenetic relationships.

METHODS

Bacterial strains and cultivation. The bacterial strains examined in this study were isolated from grasses as described previously (Behrendt *et al.*, 1997). One of the isolates (P 294/08) is deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) as DSM 11363^T. The type strains of *Sphingomonas paucimobilis*, *Sphingomonas parapaucimobilis*, *Pseudomonas mendocina* and a *Xanthomonas campestris* strain were used for a comparison of some phenotypic characteristics. The histories and corresponding numbers of the bacteria are listed in Table 1. In general, all strains were cultured on nutrient agar II (Merck) supplemented with 1.5 g yeast extract l⁻¹ and 5.0 g glucose l⁻¹ at 25 °C unless otherwise stated. Stocks of all cultures were maintained at –81 °C in nutrient broth II (SIFIN) containing 40% glycerol.

Morphological and physiological tests. Cell morphology was determined on cultures grown for 24–48 h. Cultures grown in nutrient broth at 15, 20 and 30 °C were examined microscopically for evidence of motility. Staining of flagella was performed using the method of Rudolph & Marvidis (1990). The Gram reaction was determined by three tests: classical staining procedure as described by Süßmuth *et al.* (1987), the rapid KOH string test (Ryu, 1938) and by presence of L-alanine aminopeptidase tested using Bactident test strips (Merck).

Production of oxidase was assayed on an early growth-phase culture with the aid of Bactident oxidase test strips (Merck) and the activity of catalase was tested by suspending a loopful of cells in a 10% (v/v) H₂O₂ solution. The ability to oxidize and ferment glucose was examined using the method described by Hugh & Leifson (1953). Levan formation was tested on nutrient agar supplemented with 5% sucrose. Production of a fluorescent pigment was tested on King medium B (King *et al.*, 1954). The method of Thornley (1960) was used to determine the presence of arginine dihydrolase, but was modified by covering the inoculated broth with sterile mineral oil (BioMérieux). Casein hydrolysis was assayed as described by Smibert & Krieg (1994). Denitrification was determined as described by Azegami *et al.* (1987). DNase activity was tested on DNase agar (BioMérieux) by pouring 1 M HCl onto the incubated plates. Haemolysis was tested for on Merckoplate blood agar (6.5% sheep blood) from Merck.

Utilization of carbon sources was detected by using the BIOLOG identification system. Each strain of the grass isolates was tested three times with GN plates (BIOLOG)

according to the manufacturer's recommendations. Additionally, API 20NE and API 20E tests (BioMérieux) were used to determine physiological and biochemical characteristics.

For pigment analysis, strains to be tested were grown for 3 d. Approximately 150 µl aggregated cells were scraped from the agar surface and placed in test tubes. Methanol (3 ml) was added and the mixture incubated at 50 °C for 5 min to extract methanol-soluble pigments. After centrifugation, the absorbance of the supernatant was examined using a UV/visible spectrophotometer (Uvikon 930) with methanol as a blank. The methanol extract was also examined by TLC (Schaad & Stall, 1994).

Determination of chemotaxonomic characteristics. For analysis of methylated fatty acids the strains were grown at 28 °C on Trypticase Soy agar (Merck). After 24 h growth approximately 50 mg (wet wt) of cells was harvested from the most dilute quadrant exhibiting confluent growth. Fatty acid methyl esters (FAMES) were extracted and prepared by the method described by Stead (1988).

FAMES were separated by gas chromatography and identified by mass spectrometric analysis (Saturn 3 Fa. Varian, Walnut Creek Californian; equipped with an auto-sampler 8200). Separation of FAMES was carried out on a 30 m DB-5MS column (0.25 mm inner diameter and 0.25 µm film thickness). The column oven temperature was maintained at 100 °C for 3 min and then increased by 4 °C min⁻¹ to 250 °C, followed by 10 °C min⁻¹ until 290 °C was reached, where the temperature was maintained for 6 min. The injector temperature was 260 °C. The transfer line was kept at 240 °C and the iontrap at 180 °C. The carrier gas was helium. One microlitre of the FAME preparation in tertiary methyl butyl ether/methanol solution (10:1 v/v) was injected using an autosampler.

The cells used for ubiquinone analysis and DNA extraction were obtained by cultivation at 28 °C for 48 h (24 h for ubiquinones) in 100 ml flasks containing 20 ml medium 79 (Prauser & Falta, 1968) shaken at 220 r.p.m. DNA was isolated and its G+C content determined by HPLC as described by Martin *et al.* (1997). Ubiquinones were extracted according to the method described by Tindall (1990) and separated by HPLC as described previously (Groth *et al.*, 1996). They were identified by mass spectrometry using a model GCMS-QP2000 mass spectrometer (Shimadzu) fitted with a direct sample inlet system QP 2000 DI.

PCR and restriction analysis of 16S rDNA fragments. For template preparation, single colonies grown on solid medium were picked off, resuspended in 20 µl 25 mM NaOH, 0.25% SDS and heated for 15 min at 95 °C. From this lysate 0.2 µl was directly used for PCR without further purification. Using primers fD1 and rD1 (Weisburg *et al.*, 1991), almost the complete 16S rDNA could be amplified. PCR was performed as described by Ulrich & Müller (1998). PCR products (2–8 µl) were digested with each of the following enzymes: *Cfo*I, *Hae*III, *Alu*I, *Hinf*I, *Msp*I, *Sau*3A and *Scr*FI (Boehringer, Mannheim and New England Biolabs). To detect small differences between bands, the DNA fragments were separated on 2–3.5% Metaphor agarose gels (FMC Bioproducts), depending on the fragment sizes to be distinguished.

16S rDNA sequence determination and analysis. The PCR product of the isolate P 294/08 was purified by using the QIAquick PCR purification kit (Qiagen) and digested with

Table 1. Bacterial strains used in this study

Species/ isolate	DSM no.*	Other no.†	Source	Date of isolation
P 200/02‡			Bulked sample of grasses§	20/04/93
P 217/01‡			Bulked sample of grasses§	04/05/93
P 226/02‡			Bulked sample of grasses§	11/05/93
P 257/01‡			Bulked sample of grasses§	25/05/93
P 259/20‡			Bulked sample of grasses§	25/05/93
P 265/08‡			Bulked sample of grasses§	01/06/93
P 265/09‡			Bulked sample of grasses§	01/06/93
P 274/03‡			Bulked sample of grasses§	08/06/93
P 275/05‡			Bulked sample of grasses§	08/06/93
P 278/03‡			Bulked sample of grasses§	08/06/93
P 290/01‡			Bulked sample of grasses§	22/06/93
P 294/05‡			Bulked sample of grasses§	22/06/93
P 294/08‡	11363 ^T		Bulked sample of grasses§	22/06/93
P 312/01‡			Bulked sample of grasses§	15/06/93
P 334/03‡			Bulked sample of grasses§	29/06/93
P 350/05‡			Bulked sample of grasses§	06/07/93
P 365/07‡			Bulked sample of grasses§	13/07/93
P 368/02‡			Bulked sample of grasses§	13/07/93
P 375/03‡			Bulked sample of grasses§	13/07/93
<i>Pseudomonas agarici</i>	11810 ^T	ATCC 25941 ^T	Mushroom	
<i>Pseudomonas mendocina</i>	50017 ^T	ATCC 25411 ^T	Soil	
<i>Pseudomonas oryzae</i>	6835 ^T	ATCC 43272 ^T , IAM 1568 ^T , JCM 2952 ^T	Rice paddy	
<i>Pseudomonas putida</i>	6899		Unknown	
<i>Sphingomonas parapaucimobilis</i>	7463 ^T	JCM7510 ^T	Urine	
<i>Sphingomonas paucimobilis</i>	1098 ^T	ATCC 29837 ^T , NCTC 11030 ^T	Hospital respirator	
<i>Xanthomonas campestris</i>	1050	ICPB XC 135, NCPB 1929, PDDCC 12	Unknown	

*DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

†ATCC, American Type Culture Collection, Manassas, VA; IAM, Institute of Applied Microbiology, University of Tokyo, Bunkyo-ko, Tokyo, Japan; ICPB, International Collection of Phytopathogenic Bacteria, Davis, CA, USA; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Saitama, Japan; NCPB, National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Harpenden, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; PDDCC, Plant Division Culture Collection, New Zealand Department of Scientific and Industrial Research, Auckland, New Zealand.

‡Number of culture in the collection at the Institute of Microbial Ecology and Soil Biology, Centre for Agricultural Landscape and Land Use Research Müncheberg (ZALF), Paulinenaue, Germany.

§*Poa* sp., *Festuca* sp., *Elytrigia repens*.

HaeIII, *AluI* and *SmaI* (Boehringer Mannheim). The PCR product and its restriction fragments were cloned and sequenced using fluorescent standard M13 primers. Sequencing of both strands was carried out according to the manufacturer's instructions of the AutoRead sequencing kit on an ALF DNA Sequencer (Pharmacia). Fragments of 1183–1350 nt of the generated rDNA sequence and those of the reference strains were aligned by CLUSTAL method (LaserGene, DNASTAR). ARB software [version 2.5b (1997) Technical University of Munich] was used for Felsenstein distance calculations. Dissimilarities were presented in a neighbour-joining dendrogram (Saitou & Nei, 1987).

16S rDNA sequence accession numbers. The accession numbers of the sequences used for comparison with the sequence of strain P 294/08 were as follows: *Brevundimonas diminuta* DSM 1635, X87274; *Burkholderia cepacia* DSM 50181, X87275; *Comamonas testosteroni* ATCC 11996^T, M11224; *Pseudomonas aeruginosa* DSM 50071^T, X06684; *Pseudomonas agarici* ATCC 25941^T, D84005; *Pseudomonas alcaligenes* IAM 12411^T, D84006; *Pseudomonas amygdali* ATCC 33614^T, D84007; *Pseudomonas anguilliseptica* NCIMB 1949^T, X99540; *Pseudomonas asplenii* LMG 2137^T, Z76655; *Pseudomonas avellanae* P90, U49384; *Pseudomonas azotoformans* IAM 1603^T, D84009; *Pseudomonas balearica* SP 1402^T, U26418; *Pseudomonas caricapapayae* ATCC

33615^T, D84010; *Pseudomonas chlororaphis* IAM 12354^T, D84011; *Pseudomonas cichorii* LMG 2162^T, Z76658; *Pseudomonas citronellolis* DSM 50332^T, Z76659; *Pseudomonas corrugata* ATCC 29736^T, D84012; *Pseudomonas fluorescens* NCPPB 3063^T, U01916; *Pseudomonas fluorescens* IAM 12022^T, D84013; *Pseudomonas fragi* IAM 12402, D84014; *Pseudomonas fulva* IAM 1529^T, D84015; *Pseudomonas lemoignei* LMG 2207^T, X92555; *Pseudomonas luteola* IAM 13000^T, D84002; *Pseudomonas marginalis* LMG 2210^T, Z76663; *P. mendocina* ATCC 25411^T, M59154; *Pseudomonas mucidolens* IAM 12406^T, D84017; *Pseudomonas nitroreducens* IAM 1439^T, D84021; *Pseudomonas oleovorans* IAM 1508^T, D84018; *Pseudomonas oryzihabitans* IAM 1568^T, D84004; '*Pseudomonas pavonaceae*' IAM 1155, D84019; *Pseudomonas pseudoalcaligenes* LMG 1225^T, Z76666; *Pseudomonas putida* DSM 6899, L37365; *Pseudomonas resinovorans* LMG 2274^T, Z76668; *Pseudomonas rhodesiae* CIP 104664^T, AF064459; *Pseudomonas stanieri* NCIMB 2040, X92176; *Pseudomonas straminea* IAM 1598^T, D84023; *Pseudomonas stutzeri* CCUG 11256^T, U26262; *Pseudomonas synxantha* IAM 12356^T, D84025; *Pseudomonas syringae* ATCC 19310^T, D84026; *Pseudomonas taetrolens* IAM 1653^T, D84027; *Pseudomonas tolaasii* ATCC 33618^T, D84028; *Pseudomonas viridiflava* LMG 2352^T, Z76671; *Ralstonia solanacearum* R633, U28230; *Sphingomonas parapaucimobilis* JCM 7510^T, X72721; *Sphingomonas paucimobilis* ATCC 29837^T, U37337; *Stenotrophomonas maltophilia* ATCC 33913, M59158; *X. campestris* LMG 568-T, X95917. The nucleotide sequence data for the isolate P 294/08 has been deposited in the EMBL nucleotide sequence database under the accession number Y11150.

DNA-DNA hybridization analysis. DNA-DNA homology was tested between the isolates P 265/09 and P 200/02, and also between P 294/08 (DSM 11363^T) and P 294/05, *Pseudomonas oryzihabitans*, *Pseudomonas putida* or *Pseudomonas agarici*, according to the method described by Martin *et al.* (1997).

RESULTS

Morphological, physiological and biochemical characteristics

All of the grass isolates studied were Gram-negative, aerobic, non spore-forming, rod-shaped organisms

(0.5–1.0 × 3.5–5.0 µm) that usually occurred as single cells. The cells were motile and possessed one polar flagellum, but motility depended on the temperature of incubation. They were motile when grown at 15 and 20 °C but not at 30 °C.

The optimum growth temperature was 25 °C. Cells did not grow at 42 °C but growth was slow at 4 °C. The cells did not produce a fluorescent pigment. Colonies that were 24 h old were translucent, pale yellow and convex with an entire margin. Older colonies became deep yellow. Methanol-soluble pigments extracted from whole cells of each of the grass-associated isolates, as well as from the reference strains, showed that the absorbance spectra were identical to those detected for *Sphingomonas paucimobilis* and *Sphingomonas parapaucimobilis*. Similar spectra obtained for the *Sphingomonas* species have already been described by Holmes *et al.* (1977) and Pollock (1993). A representative spectrum for each species included a major peak at 448–450 nm, a secondary peak at 474–476 nm and a slight inflection between about 415 and 430 nm. In contrast, the pigment in the methanol extract from *P. mendocina* gave only one peak, at 431 nm. *X. campestris* showed a major peak at 443 nm and two slight inflections at 417 nm and 468 nm, similar to the results of Starr *et al.* (1977). However, TLC of the methanol-soluble pigments showed different results for the R_F values of *Sphingomonas* species (0.85) in comparison to the grass-associated isolates (0.93), which indicated differences in the composition of pigments.

The rapid KOH Gram test for all grass isolates was negative. This result is in agreement with traditional Gram-stain results as well as with those for the presence of L-alanine aminopeptidase. Physiological tests which were different between the grass isolates are shown in Table 2. All strains gave positive results for catalase, aesculin hydrolysis, utilization of citrate and growth on MacConkey agar. The following characteristics were negative for all strains: fermentation and oxidation of glucose; acid production from inositol,

Table 2. Physiological tests showing differing results among the 19 strains of grass isolates tested

Test	Results of P 294/08 (DSM 11363)	No. strains (+/–)	Strains showing the less common response
Hydrolysis of gelatin	+	15/4	P 226/02; P 257/01; P 265/08; P 312/01
Casein hydrolysis	+	13/6	P 226/02; P 257/01; P 265/08; P 274/03; P 278/03; P 334/03
Oxidative acid production from:			
Melibiose	+	14/5	P 226/02; P 265/08; P 265/09; P 350/05; P 365/07
Rhamnose	–	2/16	P 259/02; P 278/03
Arabinose	+	16/3	P 265/09; P 365/07; P 375/03

sorbitol, sucrose and amygdalin; formation of levan from sucrose; oxidase reaction; reduction of nitrate to nitrite; denitrification; arginine dihydrolase; lysine decarboxylase; ornithine decarboxylase; tryptophan desaminase; DNase; urease; β -haemolysis of sheep blood; β -galactosidase; Voges–Proskauer reaction; indole production; and H₂S production from sodium thiosulphate.

Characterization of the isolates with the API 20NE system, created for the identification of non-enteric Gram-negative rods, resulted in a reaction profile which indicated an affiliation to *P. chlororaphis*. According to the appropriate API analytical profile index, the identification quality amounted to 98.5%, suggesting 'good identification'. The interpretation of results obtained with the BIOLOG Microbial Identification System suggests an identification as *P. tolaasii* at a similarity index between 0.520 and 0.646, which is also regarded as 'good identification'.

Utilization of different carbon sources estimated with the BIOLOG system are shown in Table 3. From a nutritional standpoint, the grass isolates had a broad spectrum. Many different compounds served as a sole source of carbon and energy. Preference in the utilization of carbohydrates tested was given to monomeric sugars, whilst none of the disaccharides tested were used as a substrate.

In general, the phenotypic properties of the grass-associated isolates studied were relatively homogeneous. Only a few characteristics were different between the tested strains.

Restriction analysis of amplified 16S rDNA and DNA–DNA hybridization studies among selected isolates

To analyse the heterogeneity of the 19 isolates in relation to their 16S rRNA genes, total DNA was amplified with the primers described by Weisburg *et al.* (1991). All strains showed a single band of about 1500 bp. Digestion of these PCR products with the seven endonucleases used (*Cfo*I, *Hae*III, *Alu*I, *Hin*fI, *Msp*I, *Sau*3A and *Scr*FI; a total of 36 restriction sites analysed) resulted in identical restriction patterns per enzyme for all 19 isolates (data not shown).

DNA–DNA hybridization analysis between strains P 294/08 and P 294/05 revealed a homology value of 95.9%, and 92.2% between P 265/09 and P 200/02. These results strongly support an affiliation of all grass isolates to the same species.

Chemical characteristics

The cellular fatty acid compositions of the grass isolates are shown in Table 4. In all strains, hexadecanoic acid (16:0), hexadecenoic acid (16:1) and octadecenoic acid (18:1) were the major non-polar

fatty acids, whilst the hydroxy fatty acids were represented by 3-hydroxydodecanoic acid (3-OH12:0) and 2-hydroxydodecanoic acid (2-OH12:0).

On the basis of the restriction analysis of the amplified 16S rDNA, five strains were selected for analysis of their DNA base composition and for characterization of ubiquinones. As shown in Table 5, the tested isolates contained ubiquinone Q₉ and minor amounts of ubiquinones Q₈ and Q₇. The DNA base composition ranged from 60 to 61 mol% G + C.

Phylogenetic analysis

The 16S rDNA sequence, corresponding to nucleotides 28–1524 of the *Escherichia coli* sequence, was determined for the isolate P 294/08 (DSM 11363^T). The corresponding DNA sequence has a length of 1492 bp, and was compared with 16S rDNA of 47 reference strains. The strains examined comprise species of the genus *Pseudomonas*, representing the Palleroni rRNA group I, species of *Burkholderia* and *Ralstonia* belonging to rRNA group II, and *Comamonas*, *Brevundimonas* and *Stenotrophomonas*, which are representatives of the rRNA groups III, IV and V, respectively (Palleroni, 1991a). Additionally, species of *Xanthomonas* and *Sphingomonas* were included since they belong to phenotypically similar taxa. The 16S rDNA sequence of the grass isolate showed a high level of homology to the majority of members of the genus *Pseudomonas*. Similarity values shown in Table 6 are based on data from a 1360 bp alignment. Levels of similarity to P 294/08 ranged from 64.1% to 97.7% within the genus *Pseudomonas*. The best matches were found to the 16S rDNA sequence of *P. oryzihabitans* (97.7%), *P. putida* (97.6%), *P. agarici* (97.2%), and '*P. pavonaceae*' (97.1%). In Fig. 1 an unrooted neighbour-joining dendrogram is shown for a selection of *Pseudomonas* species and some representatives of the genera mentioned above. It clearly indicates that P 294/08 is a member of the genus *Pseudomonas*. Phylogenetic analysis using the parsimony algorithm revealed a similar clustering, only the distance between *Pseudomonas* and the other genera was less distinct (data not shown).

Characterization of phylogenetic relationships to species level by 16S rDNA analysis is restricted at similarity values higher than 97% (Stackebrandt & Goebel, 1994). Thus, DNA–DNA homology was examined between P 294/08 and *P. oryzihabitans*, *P. putida* or *P. agarici*. '*P. pavonaceae*' was excluded from this study since it must be classified as an atypical strain of an invalid species (Anzai *et al.*, 1997).

The results of DNA–DNA reassociation revealed a homology value between strain P 294/08 and *P. oryzihabitans* of 18.6%, P 294/08 and *P. putida*, 26.2% and P 294/08 and *P. agarici*, 14.0% respectively. This low level of DNA homology clearly indicates a segregation of the isolate as an independent species.

Table 3. Utilization of carbon sources by grass isolates estimated with the BIOLOG system

A total of 19 strains were tested in triplicate.

Positive reaction	Negative reaction	Different reactions between strains*			
		Carbon source	+†	-†	+/-‡
Adonitol	<i>N</i> -Acetyl-D-galactosamine	D-Psicose	17§	0	2
L-Arabinose	<i>N</i> -Acetyl-D-glucosamine	Turanose	2	14	3
D-Arabitol	Cellobiose	Xylitol	14	1	4
D-Fructose	i-Erythritol	Acetic acid	15	3	1
L-Fucose	Gentiobiose	α -Hydroxybutyric acid	9	7	3
D-Galactose	α -D-Lactose	α -Ketobutyric acid	9	8	2
α -D-Glucose	Lactulose	α -Ketovaleric acid	10	7	2
<i>m</i> -Inositol	D-Melibiose	Propionic acid	11	5	3
D-Mannitol	β -Methyl-D-glucoside	Alaninamide	7	11	1
D-Mannose	D-Raffinose	Glycyl-L-glutamic acid	12	3	4
D-Sorbitol	L-Rhamnose	Hydroxy-L-proline	11	6	2
Methylpyruvate	Sucrose	L-Leucine	12	3	4
Monomethyl succinate	D-Trehalose	L-Ornithine	6	12	1
<i>cis</i> -Aconitic acid	γ -Hydroxybutyric acid	D-Serine	16	0	3
Citric acid	<i>p</i> -Hydroxyphenylacetic acid	L-Serine	16	1	2
Formic acid	Quinic acid	L-Threonine	15	1	3
D-Galactonic acid	Sebacic acid	D,L-Carnitine	17	1	1
D-Galacturonic acid	Glycyl-L-aspartic acid	D,L- α -Glycerolphosphate	14	5	0
D-Gluconic acid	L-Phenylalanine				
D-Glucosaminic acid	Thymidine				
D-Glucuronic acid	Phenylethylamine				
β -Hydroxybutyric acid	Putrescine				
Itaconic acid	2,3-Butanediol				
α -Ketoglutaric acid	Glucose-1-phosphate				
D,L-Lactic acid	Glucose-6-phosphate				
Malonic acid	α -Cyclodextrin				
D-Saccharic acid	Dextrin				
Succinic acid	Glycogen				
Bromosuccinic acid	Maltose				
Succinamic acid					
Glucuronamide					
D-Alanine					
L-Alanine					
L-Alanylglycine					
L-Asparagine					
L-Aspartic acid					
L-Glutamic acid					
L-Histidine					
L-Proline					
L-Pyroglutamic acid					
γ -Aminobutyric acid					
Urocanic acid					
Inosine					
Uridine					
2-Aminoethanol					
Glycerol					
Tween 40					
Tween 80					

* For numbers in bold type, the group contained the strain P 294/08 (DSM 11363^T).

† Number of strains which gave positive or negative results in triplicate experiments/strain.

‡ Number of strains which gave variable results in the triplicate assays.

Table 4. Cellular fatty acid composition of the grass isolates

Isolate	Fatty acid composition (%)							
	12:0	2-OH12:0	3-OH12:0	14:1	14:0	16:1	16:0	18:1
P 200/02*	2.9	0.8	2.4	0.2	0.3	57.1	27.4	8.7
P 217/01	2.0	0.3	0.5	0.1	0.3	51.6	32.1	13.2
P 226/02	3.4	0.6	1.3	0.2	—	53.1	27.4	14.0
P 257/01	3.3	2.3	2.6	0.2	0.2	57.8	28.1	5.4
P 259/20	2.5	0.4	0.9	—	—	51.1	29.5	15.6
P 265/08	—	0.2	0.9	—	—	59.9	29.3	9.6
P 265/09	2.1	0.3	0.8	—	0.2	56.5	29.7	10.4
P 274/03	2.4	0.4	0.7	—	0.2	50.1	31.7	14.5
P 275/05	2.1	0.5	0.6	0.1	0.2	60.0	31.6	4.9
P 278/03	1.0	0.2	0.4	—	—	49.9	31.0	17.6
P 290/01	2.7	0.7	0.5	0.2	—	58.0	32.8	5.0
P 294/05	2.0	0.2	0.6	—	—	50.7	31.8	14.7
P 294/08	1.9	0.2	0.6	—	—	55.6	29.8	11.9
P 312/01	3.1	0.5	—	—	—	50.4	29.7	16.2
P 334/03	0.9	0.1	—	—	—	61.2	34.3	3.5
P 350/05	2.7	0.3	0.7	—	—	51.3	31.4	13.6
P 365/07	2.4	1.0	0.8	—	0.2	54.3	30.1	11.1
P 368/02	3.2	0.4	0.8	0.1	0.3	52.2	29.9	13.1
P 375/03	—	0.1	0.5	—	—	61.4	35.5	2.4

* Also contains 0.2% 15:0.

Table 5. DNA base composition of selected grass isolates and composition of ubiquinones

Isolate	G + C content (mol %)	Ubiquinone (ratio of peak area)		
		Q ₉	Q ₈	Q ₇
P 200/02	61	66	24	4
P 265/08	60	83	3	4
P 294/05	60	76	13	3
P 294/08	60	86	9	3
P 334/03	60	69	15	2

DISCUSSION

The phenotypic characteristics of the studied grass-associated isolates were relatively homogeneous. Only a few features, which mainly concern the utilization of carbohydrates, were different between the tested strains (Tables 2, 3 and 4). These high similarities between the isolates suggest grouping as a single species. Furthermore, restriction analysis of amplified 16S rDNA and DNA–DNA reassociation studies strongly support the argument that all the strains investigated belong to the same species.

Based on this finding, chemotaxonomical classifications were focused on selected strains (Table 5). The tested isolates displayed Q₉ as the major ubiquinone,

similar to the *Pseudomonas* species of the Palleroni rRNA group I (Oyaizu & Komagata, 1983). The determined G + C content is also similar to that of members in this group (Palleroni, 1984). Physiological and morphological characteristics of the grass isolates match general phenotypic characteristics of the genus *Pseudomonas* (Palleroni, 1991a). However, the original genus *Pseudomonas* was a phylogenetically diverse assemblage of species, which could be divided into at least five distinct sub-groups based on rRNA homology data (Palleroni *et al.*, 1973). As a result of polyphasic classifications of the different rRNA groups, most species belonging to rRNA groups II–V have been transferred to other genera (Palleroni & Bradbury, 1993; Segers *et al.*, 1994; Tamaoka *et al.*, 1987; Willems *et al.*, 1989, 1990; Yabuuchi *et al.*, 1992), leaving group I as *Pseudomonas sensu stricto* (Palleroni, 1992), with *P. aeruginosa* as the type species of the genus.

For phylogenetic analysis of the strains studied, the 16S rDNA sequence determined from one of the isolates (P 294/08) was compared with the sequence of 47 reference strains representing the different rRNA groups of the original genus *Pseudomonas*, as well as phenotypically similar taxa. As shown in Fig. 1, the clear clustering to species of rRNA group I gives support to placing of the isolates within the genus *Pseudomonas sensu stricto*. The level of 16S rRNA homology between the grass isolate and the type species (91.5%) was higher than the level between

Table 6. Similarity values based on a comparison of 1340 nt of the 16S rRNA gene of the isolate P 294/08 and the corresponding sequences of proved *Pseudomonas* species

Pseudomonas species (% similarity to P 294/08)	
Similarity group I: values below 90 %	<i>P. echinoides</i> (75.6)*, <i>P. lemoignei</i> (77.2), <i>Pseudomonas stanieri</i> (64.1), <i>P. syzygii</i> (70.2)†
Similarity group II: values between 90 and 97 %	<i>P. aeruginosa</i> (91.5), <i>P. alcaligenes</i> (93.9), <i>P. amygdali</i> (94.6), <i>P. anguilliseptica</i> (94.2), <i>P. asplenii</i> (96.7), <i>P. avellanae</i> (95.1), <i>P. azotoformans</i> (95.4), <i>P. balearica</i> (94.0), <i>P. caricapapayae</i> (95.9), <i>P. chlororaphis</i> (93.4), <i>P. cichorii</i> (95.3), <i>P. citronellolis</i> (94.2), <i>P. corrugata</i> (96.0), <i>P. flavescens</i> (96.4), <i>P. fluorescens</i> (95.9), <i>P. fragi</i> (96.0), <i>P. fulva</i> (95.8), <i>P. luteola</i> (95.4), <i>P. marginalis</i> (95.5), <i>P. mendocina</i> (93.9), <i>P. mucidolens</i> (95.2), <i>P. nitroreducens</i> (96.1), <i>P. oleovorans</i> (93.7), <i>P. pseudoalcaligenes</i> (96.6), <i>P. resinovorans</i> (92.2), <i>P. rhodesiae</i> (95.7), <i>P. straminea</i> (95.1), <i>P. stutzeri</i> (96.4), <i>P. synxantha</i> (96.0), <i>P. syringae</i> (95.7), <i>P. taetrolens</i> (95.4), <i>P. tolaasii</i> (96.2), <i>P. viridiflava</i> (96.6)
Similarity group III: values above 97 %	<i>P. agarici</i> (97.2), <i>P. oryzihabitans</i> (97.7), 'P. pavonaceae' (97.1), <i>P. putida</i> (97.6)

* Comparison of a 509 bp sequence of the 16S rRNA gene (*E. coli* numbering 388–923). In this region, the similarity of P 294/08 to group II and III species ranged from 89.1 % to 98.5 % and from 96.5 to 97.8 %, respectively. Therefore, *P. echinoides* was listed in group I.

† Comparison of a 291 bp sequence of the 16S rRNA gene (*E. coli* numbering 44–337). In this region, the similarity of P 294/08 to group II and III species ranged from 84.7 to 97.9 % and from 97.2 % to 97.6 %, respectively. Therefore, *P. syzygii* was listed in group I.

Fig. 1. Unrooted phylogenetic tree showing the relationship between strain P 294/08 (*P. graminis*) and members of the genus *Pseudomonas* and some related genera. The tree was calculated from a dissimilarity matrix of a 1360 bp alignment (positions 50–1394 of *E. coli* numbering) using a neighbour-joining algorithm.

some accepted members of the genus, for example *P. syringae* (90.2%) or *P. fluorescens* (90.4%), and the type species *P. aeruginosa*.

The closest phylogenetically related species were *P. oryzihabitans*, *P. putida*, *P. agarici*, and 'P. pavonaceae', with similarity values higher than 97%. How-

Table 7. Differential characteristics of *Pseudomonas graminis* and phenotypically similar species

Data from this study and Gilardi (1991), Holmes *et al.* (1986), Holmes *et al.* (1987), Holt *et al.* (1994), Oyaizu & Komagata (1983), Segers *et al.* (1994), Yabuuchi *et al.* (1990). NT, Not tested.

Characteristic	<i>Pseudomonas graminis</i>	<i>Pseudomonas mendocina</i>	<i>Pseudomonas oryzae</i>	<i>Pseudomonas luteola</i>	<i>Brevundimonas vesicularis</i>	<i>Sphingomonas parapaucimobilis</i>	<i>Sphingomonas paucimobilis</i>	<i>Xanthomonas campestris</i>
Flagella	Polar monotrichous	Polar monotrichous*	Polar monotrichous or non-motile	Multitrichous (10–12 flagella)	Polar monotrichous	Polar monotrichous	Polar monotrichous	Polar monotrichous
Growth at 4 °C	+	–	–	–	–	–	–	–
Growth at 42 °C	–	+	–	+	–	–	–	–
Oxidation of glucose	–	+	+	+	–	+	+	+
Growth on MacConkey agar	+	+	+	+	+	–	–	–
Oxidase reaction	–	+	–	–	+	–	–	–
Arginine dihydrolase	–	+	–	+	–	–	–	–
Aesculin hydrolysis	+	–	–	+	+	+	+	+
Gelatin hydrolysis	variable	–	–	+	–	–	+	+
Starch hydrolysis	–	–	–	–	–	+	+	+
DNase	–	–	NT	NT	–	–	+	+
Lipase – Tween 80	+	+	–	–	–	+	+	+
β -Galactosidase	–	–	–	+	–	+	+	+
Major cellular hydroxy fatty acid(s)	2-OH12:0 3-OH12:0	3-OH10:0 2-OH12:0 3-OH12:0	3-OH10:0† 2-OH12:0 3-OH12:0	3-OH10:0† 2-OH12:0 3-OH12:0	3-OH12:0 3-OH14:0	2-OH14:0	2-OH14:0	3-OH10:0 3-OH12:0 2-OH13:0 3-OH11:0 3-OH13:0

* Lateral flagella may also be produced under certain conditions (Holt *et al.*, 1994).

† Data from the MIS system (Microbial ID).

ever, '*P. pavonaceae*' (IAM 1155) represents an invalid species with contradictory data. Studies by Van Landschoot *et al.* (1986) revealed that the species '*P. pavonaceae*' (NCTC 10693, CCEB 533) belongs to the *Acinetobacter* rRNA branch. Therefore, the strain must be characterized as atypical and thus not representative of its species.

At homologies higher than 97 %, the use of 16S rDNA sequence analysis for the characterization of relationships between species is limited (Stackebrandt & Goebel, 1994). For this reason, DNA–DNA hybridization experiments were performed to clarify the affiliation of the grass isolates at species level. The DNA reassociation values that were found to be lower than 26 % between the grass isolate and the immediate phylogenetic neighbours unequivocally demonstrated that the grass isolates represent an individual species in comparison to the analysed taxa.

Phenotypic features are also able to demarcate the isolates from phylogenetically related species. Most members of rRNA group I are characterized by the production of fluorescent pigments. Thus, the studied grass isolates can easily be separated from them by the presence of a yellow pigment and the absence of fluorescence. On this basis they can be distinguished from the closely related species *P. putida* and *P. agarici*. *P. flavescens* and *P. straminea* are yellow-pigmented species that are also able to produce fluorescent pigments. Additional biochemical properties, such as a positive oxidase reaction of both taxa, the production of H₂S and acid from glucose, as determined for *P. straminea*, support the differentiation from the grass

isolates on the basis of phenotypic features (Palleroni, 1984; Hildebrand *et al.*, 1994).

P. oryzae, a yellow-pigmented bacterium that is closely related to the grass-associated isolate, can be distinguished from it by the ability to oxidize glucose and the absence of aesculin hydrolysis or lipases (Table 7). Furthermore, the composition of hydroxy fatty acids is a prominent feature. Analysis of cellular fatty acids of the grass isolates revealed the presence of 2-hydroxydodecanoic acid (2-OH12:0) and 3-hydroxydodecanoic acid (3-OH12:0), as shown in Table 4. Strains of rRNA group I, including *P. oryzae*, are characterized by the occurrence of fatty acids 3-hydroxydecanoic acid (3-OH10:0) and 3-hydroxydodecanoic acid (3-OH12:0) (Oyaizu & Komagata, 1983). Thus, the composition of hydroxy fatty acids are a useful feature for separating the grass isolates from most species of *Pseudomonas sensu stricto*.

So far, the phylogenetic and phenotypic comparison of the grass isolates particularly referred to *Pseudomonas* species for which rRNA-sequences were available. Species assigned to *Pseudomonas* in the Bacterial Nomenclature Up To Date (1998), edited by the DSMZ (home page <http://www.dsmz.de/dsmzhome.html>) which were not sequenced, can also be distinguished from the grass isolates. DNA-rRNA hybridization studies of the following species revealed that they belong to rRNA groups other than the 'authentic' *Pseudomonas* group I: *Pseudomonas beteli*, *Pseudomonas boreopolis*, *Pseudomonas elongata*, *Pseudomonas hibiscicola*, *Pseudomonas nautica*, *Pseudomonas pictorum* (De Ley, 1991), *Pseudomonas beijer-*

inckii (Kerstens, 1991), *Pseudomonas geniculata* (Palleroni, 1991a), *Pseudomonas huttiensis* (Palleroni, 1984), *Pseudomonas saccharophila* (Palleroni, 1991b) and *Pseudomonas woodsii* (Schroth *et al.*, 1991). The species *Pseudomonas doudoroffii*, *Pseudomonas gelidicola* and *Pseudomonas halophila* were described as halophilic marine bacteria, which can be easily distinguished by their requirements of NaCl for growth (Holt *et al.*, 1994). Another group of species not sequenced so far can be characterized by its ability to produce a fluorescent pigment, or by the absence of a yellow pigment. Additionally, the distinction from the grass isolates is supported by several biochemical or chemotaxonomic features. This group incorporated the following species, which were able to fluoresce: *Pseudomonas aurantiaca* (Holt *et al.*, 1994), *Pseudomonas flectens* (Johnson, 1956), *Pseudomonas fuscovaginae* (Miyajima *et al.*, 1983), *Pseudomonas monteilli* (Elomari *et al.*, 1997), *Pseudomonas lundensis* (Molin *et al.*, 1986), *Pseudomonas savastanoi* (Gardan *et al.*, 1992), *Pseudomonas veronii* (Elomari *et al.*, 1996), and species that were colourless or formed white colonies [*Pseudomonas antimicrobica* (Attafuah & Bradbury, 1989), *Pseudomonas carboxydohydrogena*, *Pseudomonas ficuserectae*, *Pseudomonas iners*, *Pseudomonas pertucinogena* (Holt *et al.*, 1994), *Pseudomonas lanceolata* (Leifson, 1962b), *Pseudomonas meliae* (Oigimi, 1977), *Pseudomonas mephitica* (Haynes & Burkholder, 1957) and *Pseudomonas spinosa* (Leifson, 1962a)].

Thus, the grass isolates can be distinguished from all validly described *Pseudomonas* species on the basis of morphological, physiological and chemotaxonomic characteristics, and the results of 16S rDNA sequence comparison, DNA-rRNA and DNA-DNA hybridization studies. Consequently, we conclude that the grass isolates deserve a separate species status, for which we propose the name *Pseudomonas graminis* sp. nov.

In Table 7 physiological and chemotaxonomical features of *P. graminis* are summarized, enabling a rapid differentiation of this new species from certain yellow-pigmented species, not necessarily closely phylogenetically related but characterized by high phenotypic similarity.

Description of *Pseudomonas graminis* sp. nov.

Pseudomonas graminis (gra'mi.nis. L. n. *gramen* grass, *graminis* of grass, the source of the organism).

Cells are Gram-negative, aerobic, non spore-forming, straight rods (0.5–1.0 by 3.5–5.0 µm), motile, with one polar flagellum. Colonies are yellow, glistening, moderately convex and round with entire edges. Oxidase-negative, catalase-positive and unable to oxidize and ferment glucose. Does not produce fluorescent pigments on King medium B. Hydrolyses Tween 40 and 80, aesculin, but not starch. It is negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan desaminase, DNase, urease

and β -galactosidase. Voges-Proskauer reaction is negative. Does not produce indole or hydrogen sulphide. Reduction of nitrate to nitrite and denitrification is negative. β -Haemolysis of sheep blood is negative. Does not produce levan from sucrose. Nutritionally versatile. Substrates used by all strains are: adonitol, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, α -D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-sorbitol, methylpyruvate, monomethyl succinate, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β -hydroxybutyric acid, itaconic acid, α -ketoglutaric acid, D,L-lactic acid, malonic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-proline, L-pyroglutamic acid, γ -aminobutyric acid, urocanic acid, inosine, uridine, 2-aminoethanol and glycerol. Substrates not used by any of the strains are: *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, cellobiose, i-erythritol, gentiobiose, α -D-lactose, lactulose, maltose, D-melibiose, β -methyl-D glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, quinic acid, sebacic acid, glycyl-L-aspartic acid, L-phenylalanine, thymidine, phenylethylamine, putrescine, 2,3-butanediol, glucose-1-phosphate, glucose-6-phosphate, α -cyclodextrin, dextrin and glycogen. The G + C content of the DNA is in the range 60–61 mol %. The major isoprenoid quinone is Q₉. The major nonpolar fatty acids are hexadecanoic acid (16:0), hexadecenoic acid (16:1) and octadecenoic acid (18:1), and hydroxy fatty acids are 3-hydroxydodecanoic acid (3-OH12:0) and 2-hydroxydodecanoic acid (2-OH12:0). Isolated from the phyllosphere of grasses in Paulinenau (Germany). The type strain is DSM 11363^T (= P 294/08^T).

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