Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov., two root-associated bacteria isolated from *Brassica napus* and *Arabidopsis thaliana

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Bacteria isolates phenotypically related to *Pseudomonas corrugata* have frequently been isolated from the rhizosphere of *Arabidopsis thaliana* and *Brassica napus* grown on different soils. 16S rDNA (*rrs*) gene sequencing, DNA–DNA hybridization, biochemical characterization and siderophore typing showed that these isolates belong to two different species that are distinct from other species of the genus *Pseudomonas*, including *P. corrugata*. A description of properties of these two new species is given based on the study of 16 isolates. Proposed names are *Pseudomonas brassicacearum* (10 strains studied) and *Pseudomonas thivervalensis* (6 strains studied). The type strain of *Pseudomonas brassicacearum* is CFBP 11706^T and that of *Pseudomonas thivervalensis* is CFBP 11261^T.

Keywords: *Pseudomonas brassicacearum* sp. nov., *Pseudomonas thivervalensis* sp. nov., *Arabidopsis thaliana*, *Brassica napus*, polyphasic taxonomy

INTRODUCTION

The genus *Pseudomonas* formerly consisted of phylogenetically unrelated groups of proteobacteria including more than 100 validly described species (Palleroni, 1984; Skerman *et al.*, 1989). rRNA–DNA hybridization allowed the genus to be subdivided into five rRNA groups I–V (Palleroni *et al.*, 1973). Kersters *et al.* (1996) have summarized the recent changes in the classification of the former *Pseudomonas* species, which are now classified in about 15 genera belonging to the α , β and γ subclasses of *Proteobacteria*. The species belonging to the genus *Pseudomonas sensu stricto* are now restricted to the rRNA similarity group I of Palleroni (1984), which belongs to the γ subclass

of *Proteobacteria*. The *rrs* gene sequences of strains isolated from different environments suggest that taxonomic studies might reveal new *Pseudomonas* species (Behrendt *et al.*, 1999; Boivin-Jahns *et al.*, 1995; Whyte *et al.*, 1997; Wilson *et al.*, 1996).

Pseudomonas species are frequently isolated from different ecological niches, where some may act as pathogens of animals, plants or fungi and some are beneficial free-living soil bacteria referred to as plant-growth-promoting rhizobacteria. They are also frequently isolated from polluted sites containing high levels of pollutants such as heavy metals, polychlorinated compounds and polyaromatic hydrocarbons.

Two groups of strains related to *Pseudomonas corrugata* were isolated from the rhizoplane of *Arabidopsis thaliana* and *Brassica napus* growing in different soils. To describe the intraspecific diversity of these populations and subsequently to study the molecular interactions between *A. thaliana* and these bacteria, a

Abbreviations: ARDRA, amplified rDNA restriction analysis; IEF, isoelectric focusing; pI, isoelectric values.

The GenBank/EMBL/DBJ accession numbers for the *rrs* gene sequences in this paper are AF100321–AF100323.

Table 1. Strains used in this study

Abbreviations: CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; IS, isolated strains; NT, not tested in numerical taxonomy.

Taxon	Strain	Other designation	Source of isolation*	Phenon	Subphenon	Accession no.
<i>P. brassicacearum</i>	CFBP 11874	NFM421	<i>A. thaliana</i>	1	1c	AF100322†
<i>P. brassicacearum</i>	CFBP 11875	NFM999	<i>A. thaliana</i>	1	1c	
<i>P. brassicacearum</i>	CFBP 11862	NFM001	<i>A. thaliana</i>	1	1c	
<i>P. brassicacearum</i>	CFBP 11264	DBS11	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11711	DBS16	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11267	ABS1	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11268	D5K12	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11700	ABS2	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11699	A5K3	<i>B. napus</i>	1	1a	
<i>P. brassicacearum</i>	CFBP 11706 ^T	DBK11 ^T	<i>B. napus</i>	1	1a	AF100321†
<i>P. thivervalensis</i>	CFBP 11866	MLG19	<i>A. thaliana</i>	1	1b	
<i>P. thivervalensis</i>	CFBP 11864	MLG6	<i>A. thaliana</i>	1	1b	
<i>P. thivervalensis</i>	CFBP 11863	MLG3	<i>A. thaliana</i>	1	1b	
<i>P. thivervalensis</i>	CFBP 11867	MLG39	<i>A. thaliana</i>	1	1b	
<i>P. thivervalensis</i>	CFBP 11865	MLG14	<i>A. thaliana</i>	1	1b	
<i>P. thivervalensis</i>	CFBP 11261 ^T	SBK26 ^T	<i>B. napus</i>	1	1b	AF100323†
<i>P. corrugata</i>	CFBP 10146		<i>Lycopersicon esculentum</i>	2		
<i>P. corrugata</i>	CFBP 10883		<i>Lycopersicon esculentum</i>	2		
<i>P. corrugata</i>	CFBP 10904		<i>Lycopersicon esculentum</i>	2		D84012‡
<i>P. corrugata</i>	CFBP 2431 ^T	ATCC 29736 ^T	<i>Lycopersicon esculentum</i>	2		
<i>P. fluorescens</i> biovar II	CFBP 2125	ATCC 17482		3		
<i>P. fluorescens</i> biovar IV	CFBP 3150	ATCC 12983	Soil	3		
<i>P. fluorescens</i> biovar IV	CFBP 2129	ATCC 17513	Water	3		
' <i>P. blatchfordae</i> '	CFBP 3280	ATCC 9446 ^T	<i>Phaseolus vulgaris</i>	4		
<i>P. fluorescens</i> biovar I	CFBP 2102 ^T	IAM 12022 ^T	Water reservoir	4		D84013‡
<i>Pseudomonas</i> sp.	CFBP 2022		<i>Allium sativum</i>	4		
<i>P. marginalis</i> pv. <i>alfalfae</i>	CFBP 2039	NCPBP 2644	<i>Medicago sativa</i>	5		
<i>P. marginalis</i> pv. <i>pastinacea</i>	CFBP 2038	ATCC 13889	<i>Pastinacea sativa</i>	5		
<i>P. marginalis</i> pv. <i>marginalis</i>	CFBP 3153	ATCC 17819	Pleural fluid	5		
<i>P. fluorescens</i> biovar I	CFBP 2123	ATCC 17397	Tap water	6		
<i>P. putida</i> biotype B	CFBP 3140t1	ATCC 17430	Unknown	6		
<i>P. aureofaciens</i>	CFBP 3155t1	ATCC 17415	Soil	IS		
<i>P. asplenii</i>	CFBP 3279 ^T	LMG 2173 ^T	<i>Asplenium nidus</i>	IS		
<i>P. corrugata</i> 2140§			Wheat rhizosphere	IS		Z76655‡
' <i>P. gingeri</i> '	CFB2810	LMG 5327	<i>Agaricus bisporus</i>	IS		
<i>P. tolaasii</i>	CFBP 2068 ^T	ATCC 33618 ^T	<i>Agaricus bisporus</i>	IS		
<i>P. aureofaciens</i>	CFBP 2133 ^T	DSM 6698 ^T	Clay in kerosene	IS		Z76656‡
<i>P. chlororaphis</i>	CFBP 2132 ^T	LMG 5004 ^T	Plate contaminant	IS		
<i>P. aeruginosa</i>	CFBP 2466 ^T	LMG 1242 ^T	Unknown	IS		
<i>P. fluorescens</i> biovar V	CFBP 2130	ATCC 17386	Water	IS		
<i>P. fluorescens</i> biovar III	CFBP 3149	ATCC 17559	Unknown	IS		
<i>P. caricapapayae</i>	CFBP 3204 ^T	ATCC 33615 ^T	<i>Carica papaya</i>	IS		
<i>P. cichorii</i>	CFBP 2101 ^T	LMG 2162 ^T	<i>Cichorium endivia</i>	IS		Z76658‡
<i>P. putida</i> biotype A	CFBP 2066 ^T	ATCC 12633 ^T	Soil	IS		
<i>P. putida</i> biotype A	CFBP 3142t1		Unknown	IS		
<i>P. putida</i> biotype A	CFBP 3143t2		Unknown	IS		
<i>P. fuscovaginae</i>	CFBP 2065 ^T	NCPBP 2289 ^T	<i>Oryza sativa</i>	IS		
<i>P. agarici</i>	CFBP 2063 ^T	LMG 2112 ^T	<i>Agaricus bisporus</i>	IS		
<i>P. flectens</i>	CFBP 3281 ^T	ATCC 12775 ^T	<i>Phaseolus vulgaris</i>	IS		Z76652‡
<i>P. marginalis</i> pv. <i>marginalis</i>	CFBP 1387 ^T	LMG 2210 ^T	<i>Cichorium intybus</i>	NT		
Genomospecies FP1	CFBP 10529 ^T		<i>Lycopersicon esculentum</i>	NT		
Genomospecies FP2	CFBP 10530 ^T		<i>Lycopersicon esculentum</i>	NT		
Genomospecies FP3	CFBP 10569 ^T		<i>Lycopersicon esculentum</i>	NT		

* *A. thaliana* rhizoplane isolates were from plants growing in soils from Thiverval-Grignon, France (MLG series) or Méréville, France (NFM series). *B. napus* rhizoplane isolates were from plants growing in soils from Dieulouard, France (DB and D5 series), Andilly, France (AB and A5 series), Sexey-les-Bois, France (SBK26^T).

† EMBL/GenBank/DBJ accession number of sequences from this study.

‡ EMBL/GenBank/DBJ accession number of retrieved sequences.

§ Provided by M. H. Ryder, CSIRO Land and Water, PMB 2, Glen Osmond 5064, South Australia.

polyphasic taxonomy was necessary. Here, the description of two new *Pseudomonas* species is reported, for which the names *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov. are proposed.

METHODS

Bacterial strains and culture conditions. In total, 53 strains were studied (Table 1). A total of 16 strains was isolated, eight from the rhizosphere of *Brassica napus* cv. Brutor grown in three soils (Andilly, Sexey-les-Bois and Dieulouard, France) and eight from that of *Arabidopsis thaliana* ecotype WS grown in two soils (Thiverval-Grignon and Méréville, France). Plants were grown in pots and incubated in a greenhouse for 3 weeks (*B. napus*) or 6 weeks (*A. thaliana*). These bacteria were isolated from plant rhizoplane as previously described (Mavingui *et al.*, 1992). A semi-selective medium derived from that of Gould *et al.* (1985) was used for the isolation of strains from *A. thaliana* rhizoplane. This modified medium (RCS) contained mineral solution RCV (Heulin *et al.*, 1987) supplemented with Casamino acids (5 g l⁻¹), sodium lauroyl sarcosinate (1.2 g l⁻¹), sucrose (20 g l⁻¹) and agar (15 g l⁻¹). For strains isolated from *B. napus* rhizoplane, mineral solution RCV supplemented with sucrose (20 g l⁻¹) and agar (15 g l⁻¹) was used. These strains represented up to 10% of the total cultivable microflora associated with *A. thaliana* roots, and up to 60% of that associated with *B. napus* roots. Thirty-seven identified *Pseudomonas* strains, including 18 type strains, were used as references in the numerical taxonomy and for DNA–DNA hybridization. Strains were grown in tryptic soy broth (TSB; Difco) medium at 28 °C. They were grown for 48 h for DNA extraction and up to OD₅₉₀ 0.5 for PCR amplification. All the strains were maintained on tenfold diluted tryptic soy agar (TSA).

Conventional tests. The presence of oxidase, gelatinase and arginine dihydrolase activities, production of fluorescent pigment on King B medium, levan formation, acidification of sucrose, sorbitol, erythritol and mannitol, reduction of nitrate, hydrolysis of Tween 80 and aesculin, and the hypersensitivity reaction on tobacco leaves were tested as described by Leliott *et al.* (1966). Pectinolytic activity was tested by the method of Prunier & Kaiser (1964) and Hildebrand (1971). Utilization of lactate, L(+)-tartrate and D(–)-tartrate was tested on the basal medium of Ayers *et al.* (1919) supplemented with 0.1% (w/v) organic acid sodium salts. The presence of DNase was tested on DNA agar (Diagnostic Pasteur). Flagella were stained by the method of Rhodes (1958).

Metabolic profiles. The oxidation of 95 carbon substrates was determined using BIOLOG GN microplates as recommended by the manufacturer. Results were read with an ELISA microplate reader (Dynatech, MR5000) after 24 or 48 h incubation at 28 °C. Additionally, strains were studied for the assimilation of 99 organic substrates using Biotype 100 strips (bioMérieux) as recommended by the manufacturer. Results were read visually after 2, 3 and 4 d incubation at 26 °C.

Numerical taxonomy. A total of 114 characters, including the results of 19 conventional tests, and 95 oxidation activities obtained with BIOLOG GN microplates were included in the numerical taxonomy analysis. The distance matrix was calculated using the Jaccard coefficient (Sneath & Sokal,

1973). Cluster analysis was performed using the unweighted pair group method with averages (UPGMA). At a given distance level and for the different phenons, the amount of information for each test was measured by calculating the coefficient of diagnostic ability to determine the discriminating biochemical characteristics (Descamps & Véron, 1981).

Determination of the *rrs* gene sequences. The *rrs* gene was amplified as described in detail by Achouak *et al.* (1999). The PCR products from three rhizoplane strains (CFBP 11706^T, CFBP 11874 and CFBP 11261^T) were purified on a 1% low-melting-point agarose gel. They were directly sequenced using a protocol described by Anderson *et al.* (1992) and the sequencing primers as described by Achouak *et al.* (1999). Sequences were obtained with the Genomix system (Beckman).

Amplified rDNA restriction analysis (ARDRA). The amplified *rrs* genes were analysed by restriction digestion with 11 restriction enzymes (*AluI*, *CfoI*, *DdeI*, *HinfI*, *TaqI*, *HaeIII*, *MspI*, *RsaI*, *NdeII*, *NciI* and *ScrFI*). The restriction profiles were compared with those of the type strains of related species of *Pseudomonas*: *Pseudomonas agarici*, '*Pseudomonas blatchfordae*', *Pseudomonas flectens*, *Pseudomonas fluorescens*, *Pseudomonas fuscovaginae*, *Pseudomonas marginalis* (data from this work, Table 1); and *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *Pseudomonas cichorii*, *P. corrugata*, *P. marginalis*, *Pseudomonas mendocina*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas syringae*, *Pseudomonas tolaasii* and *Pseudomonas viridiflava* (data from Laguerre *et al.*, 1994).

Phylogenetic analysis. The *rrs* gene sequences from the three rhizoplane strains (CFBP 11706^T, CFBP 11874 and CFBP 11261^T) were aligned and analysed by comparison with a database of previously aligned sequences (about 10000). Three phylogenetic methods were used to assess the phylogenetic positions of these sequences, a neighbour-joining algorithm according to Saitou & Nei (1987), maximum-likelihood (options QFYG, FDNAML program of G. J. Olsen, University of Illinois, Urbana, IL, USA) and maximum-parsimony (PAUP 3.0s for Macintosh, heuristic search; Swofford, 1990). The robustness of each topology was checked using the neighbour-joining method and 500 bootstrap replications (Kimura two-parameters correction). Trees were drawn using the NJPLOT program for Macintosh (M. Gouy, CNRS UMR 5558, Université Claude Bernard, Lyon, France) and MACDRAW software. Domains used to construct the tree shown in Fig. 2 were regions of the *rrs* gene sequences available for all sequences: positions 49–1317 corresponding to the *rrs* gene sequence of *P. corrugata*.

DNA–DNA hybridization. Extraction and purification of DNA were performed using previously described methods (Brenner *et al.*, 1982). Native DNAs were labelled *in vitro* by nick translation with tritium-labelled nucleotides (Amersham). The S1 nuclease–trichloroacetic acid method for hybridization has been previously described (Croza *et al.*, 1973; Grimont *et al.*, 1980). The reassociation temperature was 70 °C. DNA–DNA hybridization tests were carried out using labelled DNA from strains CFBP 11261^T, CFBP 11699 and *P. corrugata* CFBP 2431^T. The strains used in DNA–DNA hybridization are listed in Table 3.

Thermal stability of DNA reassociation. The temperature at which 50% of reassociated DNA became hydrolysable by nuclease S1 (*T_m*) was determined using the method of Croza

Fig. 1. Dendrogram of phenotypic distances for 49 strains.

et al. (1973). The T_m was determined when the percentage of DNA–DNA hybridization was between 50 and 65%.

DNA base composition. The G + C contents of strains CFBP 11699 and CFBP 11261^T were determined by the thermal denaturation temperature (Marmur & Doty, 1962) and were calculated using the equation of Owen & Lapage (1976). *Escherichia coli* strain K12 CIP 54-117 (DNA G + C content 50.6 mol%) was used as a control.

Electrophoretic characterization of pyoverdines. Pyoverdines produced during growth in CAA medium [containing (l⁻¹): Bacto Casamino Acid (Difco), 5 g; K₂HPO₄ · 3H₂O, 1.54 g; MgSO₄ · 7H₂O, 0.25 g] were analysed by isoelectric focusing (IEF) according to Koedam *et al.* (1994). A Bio-Rad model 111 Mini IEF Cell apparatus was used under electrophoresis conditions as recommended by the manufacturer (15 min at 100 V, 15 min at 200 V and 1 h at 450 V). Polyacrylamide gel (5%) containing ampholines (Biolyte 3/10 from Bio-Rad) was loaded with 1 µl sample of 20-fold concentrated pyoverdine-containing CAA supernatants. Pyoverdine bands were

visualized under UV light and their corresponding isoelectric values (pI) were determined from a standard curve obtained by slicing the gel immediately after electrophoresis into 0.5 cm bands, which were incubated separately in 2 ml KCl solution (10 mM) for 30 min before measuring the pH. Electrophoresis of pyoverdines on CelloGel was performed as previously described (Meyer & Abdallah, 1978).

RESULTS

Phenotypic analysis

The dendrogram showing phenotypic distances between the 49 strains is depicted in Fig. 1. At a distance level of 0.18, six phenons and 18 isolated phenotypes were observed (Table 1). All the strains isolated from the rhizoplane of *B. napus* and *A. thaliana* clustered in phenon 1. At lower distance levels of 0.14 and 0.11, phenon 1 could be subdivided into three subphenons

Table 2. Phenotypic and biochemical characters that differentiate subphenons 1a, 1b and 1c from phenons 2–6 and from type strains of *Pseudomonas* species

+, Positive; –, negative; D, variable (number of positive strains in parentheses).

Character	Phenon or subphenon no.*								Type strains from CFBP collection†									
	1a	1b	1c	2	3	4	5	6	3279 ^T	2068 ^T	2133 ^T	2132 ^T	2466 ^T	2101 ^T	2066 ^T	2065 ^T	2063 ^T	3281 ^T
Production of pyoverdine	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan production	+	+	+	–	D(1)	D(1)	+	D(1)	–	–	+	+	–	–	–	–	–	+
DNase	+	+	+	D(2)	–	D(1)	+	–	–	+	+	+	+	–	–	–	–	–
Nitrate reduction	D(5)	D(2)	+	+	+	–	+	+	–	–	–	+	+	–	+	–	–	–
Gelatin hydrolysis	+	+	+	D(2)	–	D(2)	+	–	+	+	+	+	+	–	–	+	–	–
Assimilation of:‡																		
Adonitol	–	–	–	–	–	+	D(1)	D(1)	–	+	–	–	–	–	–	–	–	–
Erythritol	–	–	–	–	–	D(2)	+	–	–	+	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	D(2)	+	–	–	+	–	–	–	–	–	–	–	–
Sorbitol	+	+	+	–	+	+	+	+	–	+	–	+	–	+	–	–	–	–
Oxidation of:§																		
<i>p</i> -Hydroxy phenylacetic acid	+	+	+	+	–	D(2)	D(2)	+	+	–	+	+	+	–	+	+	–	+
Itaconic acid	–	–	D(1)	–	D(1)	+	+	+	–	+	+	+	+	–	–	–	+	+
γ -Hydroxybutyric acid	+	+	+	D(1)	–	–	D(1)	–	–	–	–	–	–	–	–	–	+	–
Glucuronamide	–	D(3)	D(1)	+	D(2)	+	+	D(1)	+	+	–	–	–	–	+	+	–	–
Succinamic acid	+	–	+	D(3)	+	+	+	+	–	+	+	+	+	+	+	–	+	+

* Species in each phenon or subphenon: subphenon 1a, *P. brassicacearum* ($n=7$); subphenon 1b, *P. thivervalensis* ($n=6$); subphenon 1c, *P. brassicacearum* ($n=3$); phenon 2, *P. corrugata* ($n=4$); phenon 3, *P. fluorescens* ($n=3$); phenon 4, *P. fluorescens* ($n=3$); phenon 5, *P. marginalis* ($n=3$); phenon 6, *P. fluorescens/P. putida* ($n=2$).

† *Pseudomonas* type strains are: *P. asplenii* CFBP 3279^T, *P. tolaasii* CFBP 2068^T, *P. aureofaciens* CFBP 2133^T, *P. chlororaphis* CFBP 2132^T, *P. aeruginosa* CFBP 2466^T, *P. cichorii* CFBP 2101^T, *P. putida* CFBP 2066^T, *P. fuscovaginae* CFBP 2065^T, *P. agarici* CFBP 2063^T and *P. flectens* CFBP 3281^T.

‡ Biotype 100 substrates.

§ Biolog substrates.

1a, 1b and 1c. Phenon 1 is phenotypically related to phenon 2, which contains *P. corrugata* strains, and can be differentiated by two characters: production of levan and assimilation of sorbitol (Table 2). However, phenon 1 can be clearly differentiated from the other phenons (3–6) and from the type strains of major fluorescent, oxidase-positive *Pseudomonas* species by several biochemical characters (Table 2). The biochemical character that allows discrimination of subphenons 1a, 1b and 1c is the oxidation of succinamic acid. On the basis of additional results obtained with Biotype 100 strips (data not shown), none of the 99 assimilation tests was found to discriminate among subphenons 1a, 1b and 1c.

ARDRA

The PCR products of the *rrs* gene of the 16 root-associated strains of phenon 1 and of the type strains of 20 *Pseudomonas* species, including *P. corrugata* (listed in Methods) were mapped with 11 restriction enzymes (ARDRA). The 16 root-associated strains of phenon 1 provided exactly the same profile with the restriction enzymes tested and belonged to a single ARDRA group. They showed differences with at least two restriction enzymes from the type strains of the 19 *Pseudomonas* species analysed and only *TaqI* enzyme could discriminate them from the type strain of *P. corrugata*.

rrs gene sequence analysis

Results of phylogenetic analysis clearly showed that the three strains CFBP 11706^T, CFBP 11261^T and CFBP 11874, which belong to subphenons 1a, 1b and 1c, respectively, of phenon 1 (Fig. 1), should be included in the true genus *Pseudomonas* (data not shown). To obtain a precise phylogenetic identification, analyses were thus restricted to members of this taxon, including one sequence for each validated *Pseudomonas* species and the *rrs* gene sequence of the root-associated strains. All three phylogenetic analyses (neighbour-joining, parsimony, maximum-likelihood) showed that the three rhizoplane strains formed a robust monophyletic unit (Fig. 2). Strains CFBP 11706^T, CFBP 11261^T, CFBP 11874 and *P. corrugata* formed a clade identified by all three methods that showed a high bootstrap percentage (90%). *rrs* sequence similarities were: 99.8% for CFBP 11706^T and CFBP 11874; 99.3% for CFBP 11706^T and CFBP 11261^T; 99.5% for CFBP 11261^T and CFBP 11874; 99.1% for CFBP 11706^T and *P. corrugata* ATCC 29736^T; and 99.2% for CFBP 11261^T and ATCC 29736^T.

DNA–DNA hybridization

Labelled DNA of strains CFBP 11261^T, CFBP 11699 and CFBP 2431^T was hybridized to DNA of strains of

Fig. 2. Unrooted phylogenetic tree of *Pseudomonas* 16S rDNA sequences. This topology was obtained using all known complete or almost complete sequences from *Pseudomonas* strains. The topology was obtained by neighbour-joining (Kimura two-parameters, percentage of 500 bootstrap replications shown by major branches). Asterisks indicate clades also found by maximum-likelihood ($P < 0.01$) and in the most parsimonious tree. Bar, 1 base substitution per 100 nucleotide positions.

phenon 1 and of type strains of 15 related *Pseudomonas* species or unnamed genomospecies FP1, FP2 and FP3 (Sutra *et al.*, 1997) (Table 3). Strains of subphenons 1a

and 1c showed 70–99% DNA relatedness with strain CFBP 11699 and strains of subphenon 1b showed 73–87% DNA relatedness with strain CFBP 11261^T. Levels of similarity observed between strains of subphenons 1a and 1c and strain CFBP 11261^T were 50–62% and those between strains of subphenon 1b and strain CFBP 11699 were 53–59%. However, ΔT_m values were greater than 5 °C (5.7–7.5 °C) (Table 3).

The levels of DNA relatedness of strain CFBP 11699 with the type strains of the 15 *Pseudomonas* species and genomospecies tested ranged from 9% with *P. aeruginosa* CFBP 2466^T to 39% with strain CFBP 10529 of genomospecies FP1, whereas the levels of DNA relatedness of strain CFBP 11261^T ranged from 10% with *P. aeruginosa* CFBP 2466^T to 47% with strain CFBP 10530 of genomospecies FP2. Levels of similarity observed with *P. corrugata* type strain CFBP 2431^T were 33% for strain CFBP 11699 and 34% for strain CFBP 11261^T. Moreover, the percentages of hybridization of labelled DNA of *P. corrugata* type strain CFBP 2431^T with strains CFBP 11267 and CFBP 11268 (subphenon 1a) and strain CFBP 11261^T (subphenon 1b) were 48, 45 and 46%, respectively (Table 3).

DNA base composition

The DNA G + C content of strains CFBP 11699 and CFBP 11261^T was 60.8 mol %.

Siderotyping

Strains belonging to subphenon 1b displayed an identical IEF pattern for the pyoverdines they produced during growth on CAA medium. The three bands observed for each strain of subphenon 1b were characterized by pI values of 7.5, 7.2 and 5.7, respectively (Fig. 3). In contrast, a single band with a pI of 4.0 was detected for strains belonging to subphenons 1a and 1c (Fig. 3). However, electrophoresis on CelloGel revealed three well-separated pyoverdine spots (data not shown), suggesting that the different forms (see Discussion) of the pyoverdine from phenon 1a and 1c were concentrated in a single IEF band, the migration of which was at the limit of the acid front of the pH gradient. No pyoverdine production was observed with *P. corrugata* CFBP 2431^T.

Colony morphology and phenotypic variations

At least two different colony types were observed with strains belonging to the two groups (subphenons 1a/1c and 1b) at stationary phase when grown on rich media such as LB and King B. Variants from these two groups of strains showed fluorescent pigmentation on King B medium, unlike the original isolates. The variants also showed variable characteristics for reduction of nitrate, presence of gelatinase and hydrolysis of Tween 80.

Table 3. Results of DNA–DNA hybridization experiments

NT, Not tested; IS, isolated strains.

Source of unlabelled DNA	Phenon, subphenon or isolated strain	Reassociation (%) with labelled DNA from strains:*		
		<i>P. brassicacearum</i> CFBP 11699	<i>P. thivervalensis</i> CFBP 11261 ^T	<i>P. corrugata</i> CFBP 2431 ^T
<i>P. brassicacearum</i>				
CFBP 11264	1a	98	62	NT
CFBP 11711	1a	81	59	NT
CFBP 11267	1a	89	50	48
CFBP 11268	1a	99	60	45
CFBP 11700	1a	89	60	NT
CFBP 11699	1a	100	53 (5·7)	NT
CFBP 11706 ^T	1a	96	61 (6·1)	NT
CFBP 11875	1c	95	53	NT
CFBP 11874	1c	70	50	NT
CFBP 11862	1c	86	58	NT
<i>P. thivervalensis</i>				
CFBP 11866	1b	58 (6·7)	77	NT
CFBP 11864	1b	59	82	NT
CFBP 11261 ^T	1b	56 (7·5)	100	46
CFBP 11863	1b	53	73	NT
CFBP 11867	1b	57	87	NT
CFBP 11865	1b	59	86	NT
Type strains				
<i>P. aeruginosa</i> CFBP 2466 ^T	IS	9	10	NT
<i>P. agarici</i> CFBP 2063 ^T	IS	17	15	NT
<i>P. asplenii</i> CFBP 3279 ^T	IS	30	NT	NT
<i>P. aureofaciens</i> CFBP 2133 ^T	IS	27	23	NT
<i>P. chlororaphis</i> CFBP 2132 ^T	IS	25	23	NT
<i>P. cichorii</i> CFBP 2101 ^T	IS	10	10	NT
<i>P. corrugata</i> CFBP 2431 ^T	2	33	34	100
<i>P. fluorescens</i> CFBP 2102 ^T	4	16	18	NT
<i>P. fuscovaginae</i> CFBP 2065 ^T	IS	20	17	NT
<i>P. marginalis</i> CFBP 1387 ^T	NT	NT	22	NT
<i>P. putida</i> CFBP 2066 ^T	IS	13	17	NT
<i>P. tolaasii</i> CFBP 2068 ^T	IS	24	20	NT
Genomospecies FP1 CFBP 10529 ^T	NT	39	44	NT
Genomospecies FP2 CFBP 10530 ^T	NT	29	47	NT
Genomospecies FP3 CFBP 10569 ^T	NT	13	21	NT

* Values in parentheses are values of ΔT_m in degrees Celsius.

DISCUSSION

On the basis of phenotypic data, *Pseudomonas* strains isolated from the rhizoplane of *A. thaliana* and *B. napus* clustered in a single phenon (phenon 1). All the strains were oxidase-positive, non-pectinolytic and produced levan. They produced DNase, arginine dihydrolase, and hydrolysed Tween 80 and gelatin but not aesculin. These general characteristics correspond to the characteristics of the LOPAT group Va in the determinative scheme for the fluorescent pathogenic pseudomonads of Leliott *et al.* (1966), which also includes saprophytic fluorescent *Pseudomonas* species, such as *P. fluorescens*, which are usual inhabitants of

the rhizosphere of plants. Phenon 1 is phenotypically related to phenon 2 which contains *P. corrugata* strains and can be differentiated by three characters: production of pyoverdine, production of levan and assimilation of sorbitol. Strains included in phenon 1 were phenotypically variable and distributed into three subphenons 1a, 1b and 1c. The two groups corresponding to subphenons 1a and 1c, and to subphenon 1b, respectively, can be distinguished by the assimilation of succinamic acid and, as shown in this work, by the production of different types of pyoverdine. With respect to *rrs* gene sequence analysis, strains clustered in subphenons 1a, 1b and 1c were closely related to *P. corrugata* (99.1% homology). ARDRA

Fig. 3. Isoelectric focusing of pyoverdines from *P. thivervalensis* CFBP 11261^T (a) and CFBP 11866 (b), and *P. brassicacearum* CFBP 11711 (c) and CFBP 11874 (d).

discriminated among subphenons 1a, 1b and 1c from *P. corrugata* when *TaqI* restriction enzyme was used, but failed to discriminate among isolates within these three subphenons. However, results of DNA–DNA hybridizations clearly indicated that subphenons 1a/1c and subphenon 1b belong to two discrete but closely related genomospecies, which are different from both *P. corrugata* and the other fluorescent *Pseudomonas* species tested. The DNA G + C contents of these two new genomospecies (60.8 mol%) fell within the range of G + C contents of the genus *Pseudomonas* (55.2–67.2 mol%) (De Vos *et al.*, 1989; Palleroni, 1984).

Intraclonal phenotypic variation is common in pseudomonads (Adams & Pugsley, 1934; Kelman, 1954) and has been reported recently for *P. tolaasii* (Grewal & Rainey, 1991) and *P. corrugata* (Siverio *et al.*, 1993) isolates. It results in morphologically distinct colony types originating from the same bacterial clone. In this study, strains from *A. thaliana* and *B. napus* rhizoplane, which were non-fluorescent at primary isolation, produced fluorescent variants when cultured on King B medium. The fluorescent variants appeared at the edge of non-fluorescent colonies at late stationary phase. Variants also showed variations for reduction of nitrate and hydrolysis of gelatin and Tween 80 that were different from those of the initial non-fluorescent isolates. However, pyoverdine production was identical in initial and variant strains. The role of this phenotypic variation in the ability of strains to adapt to the rhizoplane is being investigated.

In this study, the pyoverdine typing performed by IEF proved to be a powerful technique for species discrimination. Strains belonging to subphenon 1b were characterized by a single siderotype consisting of three bands with different pI values. These bands may correspond to pyoverdine forms differing in the nature

of the side-chain (succinamide, succinate, 2-oxo-glutarate, etc.) attached to the chromophore of the molecule (Budzikiewicz, 1993; Meyer *et al.*, 1997). For strains of subphenons 1a and 1c, a single IEF pattern, which was different from that of strains of subphenon 1b, was observed. An IEF–pyoverdine typing of an extended collection of bacteria is being investigated to assess the use of the method for taxonomic purposes among the fluorescent *Pseudomonas* species.

On the basis of phenotypic properties, DNA–DNA reassociation values, *rrs* gene sequencing and siderophore typing, strains included in subphenons 1a and 1c, and strains included in subphenon 1b should be considered as two new *Pseudomonas* species. The names *Pseudomonas brassicacearum* sp. nov. and *Pseudomonas thivervalensis* sp. nov. are proposed.

Description of *Pseudomonas brassicacearum* sp. nov.

Pseudomonas brassicacearum (bras.si.ca.ce.a'rum. M. L. fem. pl. gen. n. *brassicacearum* of the *Brassicaceae*, referring to its isolation from the rhizoplane of plants belonging to the *Brassicaceae*).

The studied strains belong to a larger collection of isolates that belong to the species *P. brassicacearum*. They were isolated from the rhizoplane of *A. thaliana* and *B. napus* growing on different soils. They represent more than 10% of total cultivated bacteria associated with the roots of these plants (unpublished data). Gram-negative motile rods, 1.0–1.5 µm in length, about 0.5 µm in diameter. Forms mucoid colonies with regular margins when grown on TSA and produces brown–orange diffusible pigment at late stationary phase on TSA and in TSB, but not on these media when diluted tenfold. Can also produce a fluorescent pigment when grown on CAA medium. Aerobic growth, oxidase-positive, arginine dihydrolase-positive, produces levan, not pectinolytic. Reduction of nitrate to nitrite is variable. Hydrolyses gelatin, DNA and Tween 80 but not aesculin. Produces no hypersensitivity reaction on tobacco leaves. Acid produced from sucrose and sorbitol but not from erythritol and mannitol. Results obtained with BIOLOG GN microplates indicate that strains oxidize the following substrates: acetic acid, *N*-acetylglucosamine, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, γ-aminobutyric acid, 2-aminoethanol, L-arabinose, D-arabitol, L-asparagine, L-aspartic acid, *cis*-aconitic acid, bromosuccinic acid, DL-carnitine, citric acid, formic acid, D-fructose, D-galactonic acid lactone, D-galactose, D-galacturonic acid, α-D-glucose, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, L-glutamic acid, glycerol, L-histidine, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenyl acetic acid, hydroxy-L-proline, inosine, *m*-inositol, α-ketoglutaric acid, DL-lactic acid, malonic acid, D-mannitol, D-mannose, methylpyruvate, monomethylsuccinate, L-ornithine, L-proline, propionic acid, L-pyroglutamic acid, quinic acid, D-saccharic acid, sebacic acid, L-serine, D-sorbitol, succinamic acid, succinic acid,

sucrose, D-trehalose, L-threonine, Tween 40, Tween 80, uridine and urocanic acid. Oxidation of 2,3-butanediol, DL- α -glycerol phosphate, glycyl L-glutamic acid, α -hydroxybutyric acid, α -ketobutyric acid, α -ketovaleric acid, L-leucine, maltose, psicose and turanose is variable. Oxidation of the other organic substrates included in BIOLOG GN microplates is negative. *P. brassicacearum* strains assimilate the following substrates (Biotype 100 strips): *N*-acetyl-D-glucosamine, *cis*-aconitate, *trans*-aconitate, D-alanine, L-alanine, DL- α -amino-n-butyrate, DL- α -amino-n-valerate, L-arabinose, D-arabitol, L-aspartate, betaine, caprate, caprylate, citrate, ethanolamine, β -D-fructose, fumarate, D-galactose, D-galacturonate, D-gluconate, D-glucosamine, α -D-glucose, D-glucuronate, L-glutamate, glutarate, DL-glycerate, glycerol, 2-keto-D-gluconate, 2-oxoglutarate, DL- β -hydroxybutyrate, *p*-hydroxybenzoate, DL-lactate, D-lyxose, L-malate, D-mannose, D-mannitol, mucate, *myo*-inositol, L-proline, propionate, protocatechuate, quinate, D-ribose, D-saccharate, L-serine, D-sorbitol, succinate, sucrose, D-trehalose, trigonelline, L-tyrosine and D-xylose. Assimilation of benzoate, D(+)-malate, malonate, putrescine and D(-)-tartrate is variable. The other organic substrates included in Biotype 100 strips are not assimilated. DNA G+C content is 60.8 mol%. Type strain is strain DBK11^T which has been deposited in the Collection Française de Bactéries Phytopathogènes (CFBP, Angers, France) under the number CFBP 11706^T.

Description of *Pseudomonas thivervalensis* sp. nov.

Pseudomonas thivervalensis (thi.ver.va.len'sis. M.L. adj. *thivervalensis* pertaining to Thiverval, France, the place where most of the strains have been isolated from the rhizoplane of *Arabidopsis thaliana*).

Gram-negative motile rods, 1.0–1.5 μ m in length, about 0.5 μ m in diameter. Forms mucoid colonies with regular margins when grown on TSA and produces brown–orange diffusible pigment at late stationary phase on TSA and in TSB, but not on these media when diluted tenfold. They can also produce a fluorescent pigment when grown on CAA medium. *P. thivervalensis* has the same general characteristics as *P. brassicacearum*. Oxidation of the following substrates is variable: D-galacturonic acid, glucuronamide, D-glucuronic acid, L-ornithine, putrescine and L-threonine. Unlike *P. brassicacearum*, *P. thivervalensis* strains do not oxidize succinamic acid. *P. thivervalensis* strains do not assimilate benzoate unlike 80% of *P. brassicacearum* strains. The oxidation and the assimilation of the other organic substrates tested with BIOLOG GN microplates and with Biotype 100 strips are the same for strains of *P. thivervalensis* as for *P. brassicacearum* strains (see above). DNA G+C content is 60.8 mol%. The type strain is strain SBK26^T, which has been deposited in the Collection Française de Bactéries Phytopathogènes (CFBP) under number CFBP 11261^T.

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