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Pseudomonas mosselii sp. nov., a novel species isolated from clinical specimens

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Twenty-two fluorescent pseudomonad strains of clinical origin received as *Pseudomonas fluorescens* (10 strains), *Pseudomonas putida* (10 strains) and *Pseudomonas* sp. (2 strains), and 33 type strains of the genus *Pseudomonas* were studied by numerical analysis based on 280 phenotypic characters. Twelve of the 22 clinical isolates clustered within a specific group, cluster IV. The other strains clustered within groups containing well-characterized fluorescent *Pseudomonas* species or did not cluster. Strains belonging to cluster IV were phenotypically different from all other clusters and subclusters of fluorescent pseudomonads. DNA–DNA hybridization showed that cluster IV corresponded to a genomic group sharing 72–100% DNA relatedness. DNA–DNA hybridization values with 67 strains representing 30 species of the genus *Pseudomonas sensu stricto*, including six recently described species (*Pseudomonas veronii*, *Pseudomonas rhodesiae*, *Pseudomonas libanensis*, '*Pseudomonas orientalis*', '*Pseudomonas cedrella*' and *Pseudomonas monteilii*), were below 49%, the value found for *P. monteilii*. The DNA G+C content of the type strain was 63 mol%. Comparison of the 16S rRNA gene sequence of a representative strain of cluster IV (CFML 90-83^T) with sequences of other strains of the genus *Pseudomonas* revealed that strain CFML 90-83^T was part of the *P. fluorescens* intrageneric cluster. On the basis of phenotypic, DNA–DNA hybridization and phylogenetic analyses, a novel species, *Pseudomonas mosselii* sp. nov., is proposed for the 12 strains of cluster IV. The type strain is *P. mosselii* CFML 90-83^T (= ATCC BAA-99^T = CIP 105259^T). The *P. mosselii* strains are phenotypically homogeneous and can be differentiated from other fluorescent species by several phenotypic features, including pyoverdine typing.

Keywords: *Pseudomonas*, DNA–DNA hybridization, 16S rRNA, siderotyping

INTRODUCTION

The genus *Pseudomonas* includes metabolically versatile organisms utilizing a wide range of organic compounds (Stanier *et al*., 1966). These bacteria are ubiquitous in soil and water and some are also important as plant, animal and human pathogens (Palleroni, 1992). The nomenclature of bacteria classified in the genus *Pseudomonas* has changed considerably during the last decade. By measuring the similarities of various *Pseudomonas* species by rRNA–DNA hybridization, Palleroni *et al*. (1973) were able to subdivide the genus into five distantly related so-called rRNA groups (rRNA groups I–V). Genuine *Pseudomonas* species are currently restricted to taxa belonging or related to rRNA group I of Palleroni (De Vos & De Ley, 1983; De Vos *et al*., 1989; Palleroni & Bradbury, 1993; Segers *et al*., 1994; Willems *et al*., 1992; Yabuuchi *et al*., 1992). Species of *Pseudomonas sensu lato* have been classified into several genera: *Pseudomonas sensu stricto*, *Chryseomonas*, *Flavimonas* (Holmes *et*

Abbreviations: IEF, isoelectrofocusing; K_{nuc}, nucleotide substitution rate; pH_i, isoelectric pH; SD, Dice coefficient; T_m, temperature at which 50% reassociated DNA is hydrolysed by nuclease S1; ΔT_m, difference between heteroduplex T_m and homoduplex T_m.

The EMBL accession number for the 16S rRNA gene sequence of strain CFML 90-83^T is AF072688.

Table 1. List of organisms studied and their classification based on phenotypic analysis

Culture collection or other reference number*	Name as received	Source	Cluster†	Subcluster†
NCPPB 1616	*P. tolaasii*	Cultivated mushroom *Agaricus bisporus*	I	
NCPPB 2192^T	*P. tolaasii*	Cultivated mushroom *Agaricus bisporus*	I	
ATCC 13525^T	*P. fluorescens* biovar I	Pre-filter	I	
ATCC 17563	*P. fluorescens* biovar I	Tap water	I	
CFML 90-132	*P. fluorescens*	Sputum	II	
ATCC 10844^T	*P. marginalis*	Endive	II	
DSM 50275	*P. marginalis*	Plant	II	
DSM 50276	*P. marginalis*	Soft rot of potato tuber	II	
DSM 50106	*P. fluorescens* biovar II	Sea water	II	
ATCC 12633^T	*P. putida* biovar A	Tracheal aspirate	III	IIIa
CFML 90-42	*P. putida*	Placenta	III	IIIa
CFML 90-46	*P. putida*	Sputum	III	IIIa
CFML 90-47	*P. putida*	Urine	III	IIIa
CFML 90-49	*P. putida*	Urine	III	IIIa
CFML 90-52	*P. putida*	Infected skin	III	IIIa
CFML 90-39	*P. putida*	Suppuration	III	IIIa
CFML 90-40	*P. putida*	Bronchial aspirate	III	IIIa
CIP 104883^T	*P. monteilii*	Clinical isolate	III	IIIb
CFML 90-59	*P. monteilii*	Clinical isolate	III	IIIb
CFML 90-60	*P. monteilii*	Bronchial aspirate	III	IIIb
CFML 90-61	*P. monteilii*	Bronchial aspirate	III	IIIb
CFML 90-62	*P. monteilii*	Urine	III	IIIb
CFML 90-41	*P. putida*	Urine	III	NC
CFML 90-83^T	*P. putida*	Tracheal aspirate	IV	
CFML 90-70	*P. fluorescens*	Bronchial aspirate	IV	
CFML 90-71	*P. fluorescens*	Blood culture	IV	
CFML 90-73	*P. fluorescens*	Veinous catheter	IV	
CFML 90-74	*P. fluorescens*	Stool	IV	
CFML 90-75	*Pseudomonas* sp.	Drainage liquid	IV	
CFML 90-76	*Pseudomonas* sp.	Faeces	IV	
CFML 90-77	*P. fluorescens*	Bronchial aspirate	IV	
CFML 90-78	*P. fluorescens*	Clinical isolate	IV	
CFML 90-79	*P. fluorescens*	Clinical isolate	IV	
CFML 90-80	*P. fluorescens*	Clinical isolate	IV	
CFML 90-82	*P. fluorescens*	Clinical isolate	IV	
ATCC 13985^T	*P. aureofaciens*	Mass river clay in kerosene	V	
DSM 50083^T	*P. chlororaphis*	Plate contaminant	V	
CCUG 1317	*P. putida* biovar B	Soil	VI	
ATCC 17430	*P. putida* biovar B	Soil	VI	
ATCC 10145^T	*P. aeruginosa*	Infected wound	VII	
ATCC 27853	*P. aeruginosa*	Blood culture	VII	
ATCC 17559	*P. fluorescens* biovar III	Plant	VIII	
ATCC 17571	*P. fluorescens* biovar III	Polluted sea water	VIII	
CCM 573^T	*P. lundensis*	Beef meat	IX	
CCUG 18758	*P. lundensis*	Beef meat	IX	
NCPPB 1873^T	*P. caricapapayae*	Unknown	NC	
ATCC 19310^T	*P. syringae*	Plant	NC	
CCUG 1313^T	*P. fragi*	Unknown	NC	
DSM 50259^T	*P. cichorii*	Endive	NC	
ATCC 25941^T	*P. agarici*	Unknown	NC	
ATCC 23835^T	*P. asplenii*	Plant	NC	
DSM 50148	*P. fluorescens* biovar V	Soil	NC	

Table 1 (*cont.*)

Culture collection or other reference number*	Name as received	Source	Cluster†	Subcluster†
ATCC 17815	*P. fluorescens* biovar II	Lettuce	NC	
CFML 90-65	*P. putida*	Urine	NC	
DSM 50415	*P. fluorescens* biovar IV	Unknown	NC	

*ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCPPB, National Collection of Plant-Pathogenic Bacteria, Plant Pathology Laboratory, Hatching Green, Harpenden, UK; CIP, Collection de l'Institut Pasteur, Paris, France; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyne, University, Brno, Czech Republic; CFML, Collection de la Faculté de Médecine de Lille, Lille, France.

† NC, Strain does not belong to any major phenotypic cluster or subcluster as defined by numerical analysis.

al., 1987), *Burkholderia* (Yabuuchi *et al.*, 1992), *Ralstonia* (Yabuuchi *et al.*, 1995), *Comamonas* (De Vos *et al.*, 1985), *Acidovorax* (Willems *et al.*, 1990), *Hydrogenophaga* (Willems *et al.*, 1989), *Telluria* (Bowman *et al.*, 1993), *Stenotrophomonas* (Palleroni & Bradbury, 1993), *Brevundimonas* (Segers *et al.*, 1994), *Aminobacter* (Urakami *et al.*, 1992), *Oligotropha*, *Zavarzinia* (Meyer *et al.*, 1993), *Sphingomonas* (Yabuuchi *et al.*, 1990), *Devosia* (Nakagawa *et al.*, 1996) and *Delftia* (Wen *et al.*, 1999). The genus *Pseudomonas sensu stricto* consists of both fluorescent and non-fluorescent species. The fluorescent pseudomonads are characterized by the production of water-soluble pigments, the pyoverdines, which act as powerful siderophores for these bacteria (Meyer & Stintzi, 1998). Most of the fluorescent species are saprophytic (e.g. *Pseudomonas fluorescens*) or pathogenic for humans (e.g. *Pseudomonas aeruginosa*), plants (e.g. *Pseudomonas cichorii*, *Pseudomonas marginalis*, *Pseudomonas syringae*, *Pseudomonas savastanoi*) or mushrooms (e.g. *Pseudomonas agarici*, *Pseudomonas tolaasii*).

Detailed phenotypic, chemotaxonomic or genotypic studies indicate that *P. fluorescens* and *Pseudomonas putida* are heterogeneous *Pseudomonas* species (Vancanneyt *et al.*, 1996). Strains of *P. putida* and *P. fluorescens* are very common environmental contaminants and these organisms are rarely pathogenic for humans, even though they have been found associated with urinary tract infections, septicaemia, septic arthritis, osteomyelitis, wound infections, pelvic inflammatory disease and various other diseases (Palleroni, 1992). There are also numerous references reporting their isolation from a variety of materials of clinical origin (Blazevic *et al.*, 1973; Gilardi, 1972; Pedersen *et al.*, 1970; Rogers, 1960; Sutter, 1968). In any event, *P. putida* and *P. fluorescens* should be considered potentially pathogenic (Von Graevenitz & Weinstein, 1971).

This study was intended to provide a phenotypic, genotypic and phylogenetic analysis of 22 strains of clinical origin related to *P. putida*, *P. fluorescens* and *Pseudomonas* sp. From this study, a novel species, *Pseudomonas mosselii* sp. nov., is formally described; the type strain is CFML 90-83^T (= ATCC BAA-99^T = CIP 105259^T).

METHODS

Bacterial strains. A total of 89 strains was used in this study. Twenty-two strains were isolated from clinical specimens, received as *P. putida* (10 strains), *P. fluorescens* (10 strains) and *Pseudomonas* sp. (2 strains). Sixty-seven strains were used as references for numerical taxonomy and DNA–DNA hybridization studies, representing 30 species of the genus *Pseudomonas sensu stricto* (Kersters *et al.*, 1996), including 6 newly described *Pseudomonas* species: *Pseudomonas veronii* (Elomari *et al.*, 1996), *Pseudomonas rhodesiae* (Coroler *et al.*, 1996), both isolated from French mineral waters, *Pseudomonas monteilii* (Elomari *et al.*, 1997) isolated from clinical specimens, and '*Pseudomonas cedrella*', '*Pseudomonas orientalis*' (Dabboussi *et al.*, 1999a) and *Pseudomonas libanensis* (Dabboussi *et al.*, 1999b) isolated from Lebanese spring waters (Table 1). All bacteria were cultured routinely on Mueller–Hinton medium at 30 °C.

Phenotypic characterization. Two-hundred-and-eighty phenotypic characters were determined. Forty-four conventional tests were performed as described by Gavini *et al.* (1989). Assimilation of 147 compounds, including carbohydrates, organic acids and amino acids, as sole carbon source was tested using API 50-CH, API 50-AO and API 50-AA galleries (bioMérieux); growth was observed after 2, 4 and 6 d, respectively. Eighty-nine enzymic activities, including 59 peptidase, 10 esterase and 20 oxidase activities, were tested as described previously (Gavini *et al.*, 1991). These tests were also studied with the API system (API ZYM; bioMérieux), incubated at 30 °C for 4 h. They were scored according to the manufacturer's recommendations. Tests which were either positive or negative for all the strains were not included in the numerical analysis; other data were coded 1 for positive results, 0 for negative results and subjected to numerical analysis as described by Gavini *et al.* (1989).

Numerical taxonomic analysis. Based on a matrix containing 55 strains and 230 tests, the Dice coefficient (SD) for each pair of strains was obtained as follows: $SD = 2a/(2a+b+$

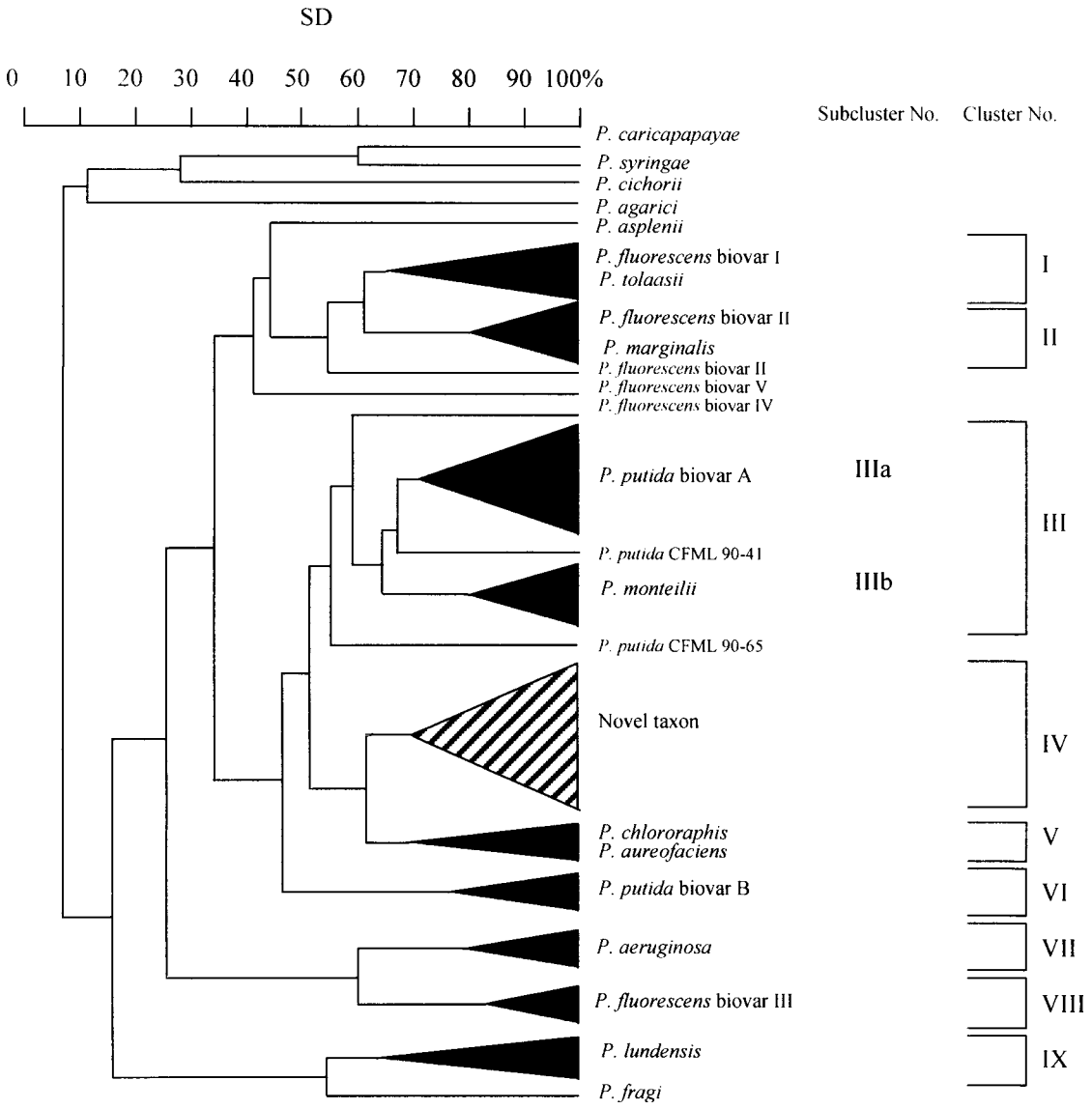

Fig. 1. Phenotypic dendrogram based on unweighted pair group average linkages. SD, Dice similarity index. The cut-off level between clusters is 62 %.

c), where '*a*' is the number of positive resemblances, and '*b*' and '*c*' are the number of divergences. Groupings were made using the unweighted pair group method using arithmetic averages. The software used for the Dice coefficient and groupings determination was HIERAR (Université des Sciences et Technologies, Lille, France).

Isolation and purification of genomic DNA. Cells were grown on Mueller–Hinton agar plates at 30 °C, then harvested and washed in 10 mM Tris buffer, pH 8 containing 1 mM EDTA and resuspended in the same buffer. Genomic DNA was extracted and purified by alkali lysis with SDS according to the method described by Beji *et al.* (1987).

DNA–DNA hybridization and thermal stability of reassociated DNAs. The relatedness of strains was determined by DNA–DNA hybridization as described by Grimont *et al.* (1980). DNA from strain CFML 90-83^T (cluster IV, proposed below as the type strain of *P. mosselii* sp. nov.) was labelled *in vitro* with ^{3}H-cytosine by nick-translation according to the manufacturer's instructions (Amersham). DNA hybridization reactions were done using the S1 nuclease/trichloroacetic acid method at 60 °C (Crosa *et al.*, 1973; Grimont *et al.*, 1980). The temperature at which 50 % reassociated DNA was hydrolysed by nuclease S1 (T_m) was determined using the method of Crosa *et al.* (1973). ΔT_m is the difference between T_m of the heteroduplex and T_m of the homoduplex.

DNA base composition. The G+C content of genomic DNA was calculated from the thermal denaturation curve using the equation of De Ley (1970). *Escherichia coli* ATCC 11775^T DNA was used as the reference (G+C content, 51 mol%).

Characterization of pyoverdines by isoelectrofocusing. Bacterial strains were grown for 40 h at 25 °C with shaking (200 r.p.m.) in CAA medium [CAA medium contained (g l^{-1}): Casamino acids (Difco), 5; HK_2PO_4, 1·2;

Table 2. Features differentiating *P. mosselii* CFML 90-83^T (= ATCC BAA-99^T = CIP 105259^T) and closely related fluorescent species and biovars of the genus *Pseudomonas*

Strains: 1, *P. mosselii*; 2, *P. monteilii*; 3, *P. fluorescens* biovar I; 4, *P. fluorescens* biovar II; 5, *P. fluorescens* biovar III; 6, *P. fluorescens* biovar IV; 7, *P. fluorescens* biovar V; 8, *P. chlororaphis*; 9, *P. putida* biovar A; 10, *P. putida* biovar B; 11, *P. veronii*; 12, *P. rhodesiae*; 13, *P. libanensis*; 14, '*P. orientalis*'; 15, '*P. cedrella*'. −, 90% or more of the strains are negative; +, 90% or more of the strains are positive; d, 11–89% of the strains are positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pyocyanin production	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Denitrification	−	−	−	+	+	+	−	+	−	−	+	−	−	−	d
Growth at 4 °C	−	−	+	+	+	+	d	+	d	+	+	+	+	+	+
Growth at 41 °C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Lecithinase	d	−	+	d	+	+	d	d	−	−	−	+	+	d	−
Gelatinase	d	−	+	+	+	+	+	+	−	−	d	−	−	−	−
Assimilation of:															
Ribose	+	+	+	+	d	+	d	+	d	d	+	+	+	+	+
D-Xylose	−	−	+	d	d	d	d	−	d	d	+	+	+	+	+
α-L-Rhamnose	d	−	−	d	d	−	d	−	−	−	−	−	d	d	d
D-Mannose	+	−	+	+	+	+	d	+	d	d	+	+	+	+	+
D-Mannitol	d	−	+	+	d	+	d	+	d	d	ND	ND	+	+	+
D-Trehalose	d	−	+	+	d	+	d	+	−	−	d	+	d	+	+
2-Keto-D-gluconate	+	+	+	+	+	d	+	+	d	+	+	+	+	+	+
Mucate	−	−	+	+	d	+	+	+	d	+	d	+	+	+	+
Malonate	−	d	+	+	d	+	d	+	d	+	+	+	+	+	+
D-Tartrate	−	−	−	d	−	−	d	−	d	d	−	−	−	−	d
meso-Tartrate	+	−	−	−	d	−	d	−	d	−	−	−	d	−	−
Erythritol	−	−	d	d	+	−	d	−	−	−	+	−	+	−	+
D-Sorbitol	d	−	+	+	d	+	d	−	−	d	+	+	+	+	+
myo-Inositol	−	+	d	+	d	+	d	+	−	−	+	+	+	+	+
Adonitol	−	−	+	−	d	−	d	−	−	−	−	−	+	+	+
Benzoate	−	+	d	d	d	+	d	ND	d	+	+	−	−	−	−
α-Aminobutyrate	+	+	−	−	−	−	−	−	−	d	+	+	+	+	+
L-Histidine	+	+	+	d	+	+	d	+	+	+	d	+	−	−	−
L-Tryptophan	−	−	+	d	d	−	d	+	−	+	+	−	−	−	−
Histamine	+	d	d	−	d	−	d	d	d	+	−	−	−	−	−
Tryptamine	−	−	−	d	d	−	−	−	d	+	−	−	−	−	−
Trigonelline	−	d	d	d	d	−	d	−	d	+	+	−	−	+	+

$MgSO_4.7H_2O$, 0·25] dispensed as 7·5 ml per 18 × 180 mm capped test-tube and the pyoverdine-containing growth supernatant was subjected to isoelectrophoresis according to Koedam *et al.* (1994). The Bio-Rad model 111 mini isoelectrofocusing (IEF) cell apparatus was used under electrophoresis conditions as recommended by the manufacturer. A polyacrylamide gel (5%) containing ampholines (BioLyte 3/10; Bio-Rad) was prepared according to the manufacturer's recommendations and loaded with 1 µl samples of 20-fold concentrated CAA growth supernatants. Supernatants were concentrated by lyophilization of 400 µl growth supernatant, the residue being dissolved in 20 µl double-distilled water. Pyoverdine bands were visualized under UV light and analysed for their corresponding isoelectric pH values (pH_i) as previously described (Meyer *et al.*, 1997).

16S rRNA gene sequence determination. The almost complete 16S rRNA gene sequence was determined for strain CFML 90-83^T by direct PCR sequencing. DNA was amplified using the 16S rRNA universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′), which are complementary to *E. coli* 16S rRNA positions 8–27 and 1544–1525, respectively (Brosius *et al.*, 1978; Edwards *et al.*, 1989; Lane, 1991). PCR amplification was performed in a model 480 DNA thermal cycler (Perkin-Elmer) using a PCR mixture (final vol. 100 µl) containing each of the four dNTP at a concentration of 200 µM, primers pA and pH at a concentration of 1 mM each, 1 µg target DNA and 2·5 U *Taq* DNA polymerase. The sequence of the PCR-amplified 16S rRNA gene was determined directly, using an Applied Biosystems 377 automated DNA sequencer according to the protocols specified by the manufacturer and the following primers: pA and pH (used for amplification); primer 59-83 (5′-CTTATTCTGTCGGTAACGTC-3′); primer 21-83 (5′-GGGCTCAACCTGGGAACTGC-3′); primer 15-83 (5′-TCCACCGCTTGTGCGGGCCC-3′); and primer 35-83 (5′-AGTTACCAGCCACGTCATGG-3′). The latter four primers covered the sequence at the following positions: 493–474, 608–627, 939–920 and 1116–1135, respectively [*E. coli* numbering (Brosius *et al.*, 1978)].

Table 3. Levels of DNA relatedness of *P. mosselii* to different type and collection strains of the genus *Pseudomonas*

Source of unlabelled DNA*	Labelled DNA from strain CFML 90-83^T	
	RBR (%)†	ΔT_m (°C)
Cluster IV		
CFML 90-83^T	100	
CFML 90-74	88	
CFML 90-78	84	
CFML 90-70	81	
CFML 90-80	80	
CFML 90-82	79	2
CFML 90-75	77	4
CFML 90-71	75	1
CFML 90-77	74	4
CFML 90-73	73	4
CFML 90-79	73	3
CFML 90-76	72	2
Cluster II		
CFML 90-132	19	
Subcluster IIIa		
CFML 90-42	18	
CFML 90-46	22	
CFML 90-47	26	
CFML 90-49	19	
CFML 90-52	25	
CFML 90-39	10	
CFML 90-40	29	
NC		
CFML 90-41	26	
CFML 90-65	41	8
P. putida biovar A ATCC 12633^T	40	9
P. putida biovar B CCUG 1317	15	
P. putida biovar B ATCC 17430	20	
P. putida biovar B ATCC 17484	22	
P. monteilii CIP 104833^T	49	10
P. aeruginosa ATCC 10145^T	20	
P. aeruginosa ATCC 27853	19	
P. aeruginosa ATCC 15692	13	
P. fluorescens biovar I ATCC 13525^T	33	
P. fluorescens biovar I ATCC 17563	20	
P. fluorescens biovar II ATCC 17816	29	
P. fluorescens biovar II ATCC 17815	22	
P. fluorescens biovar II ATCC 17482	18	
P. fluorescens biovar II DSM 50106	26	
P. fluorescens biovar III ATCC 17559	15	
P. fluorescens biovar III ATCC 17400	16	
P. fluorescens biovar III ATCC 17571	15	
P. fluorescens biovar IV DSM 50415	16	
P. fluorescens biovar IV ATCC 12983	13	
P. fluorescens biovar V ATCC 14150	25	
P. fluorescens biovar V ATCC 17518	34	
P. fluorescens biovar V ATCC 15916	16	
P. fluorescens biovar V ATCC 17386	15	
P. fluorescens biovar V ATCC 17573	18	
P. fluorescens biovar V DSM 50148	16	
P. marginalis ATCC 10844^T	19	
P. marginalis DSM 50275	22	

Table 3 (*cont.*)

Source of unlabelled DNA*	Labelled DNA from strain CFML 90-83^T	
	RBR (%)†	ΔT_m (°C)
P. marginalis DSM 50276	25	
P. libanensis CIP 105460^T	15	
'*P. cedrella*' CIP 105541^T	9	
'*P. orientalis*' CIP 105540^T	12	
P. chlororaphis DSM 50083^T	16	
P. chlororaphis ATCC 9447	33	
P. chlororaphis ATCC 17414	17	
P. aureofaciens ATCC 13985^T	31	
P. aureofaciens ATCC 17415	23	
P. veronii CIP 104663^T	32	
P. rhodesiae CIP 104664^T	36	
P. synxantha CIP 5922^T	27	
P. mucidolens CIP 103298^T	30	
P. lundensis CCM 573^T	14	
P. lundensis CCUG 18785	10	
P. syringae ATCC 19310^T	16	
P. savastanoi CFBP 1670^T	9	
P. savastanoi CFBP 2088	13	
P. savastanoi CFBP 1838	11	
P. viridiflava ATCC 13223^T	9	
P. cichorii DSM 50259^T	11	
P. agaricii ATCC 25941^T	10	
P. asplenii ATCC 23835^T	10	
P. caricapapayae NCPPB 1873^T	7	
P. tolaasii NCPPB 2129^T	24	
P. tolaasii NCPPB 1616	20	
P. stutzeri ATCC 17588^T	7	
P. stutzeri ATCC 17591	9	
P. stutzeri ATCC 17587	10	
P. stutzeri ATCC 17686	7	
P. mendocina ATCC 25411^T	7	
P. mendocina ATCC 25412	8	
P. alcaligenes ATCC 14909^T	11	
P. pseudoalcaligenes ATCC 17440^T	8	
P. pseudoalcaligenes ATCC 12815	9	
P. fragi CCUG 1313^T	10	
P. fragi ATCC 27362	11	
P. flavescens NCPPB 3063^T	19	
P. flavescens CIP 104205	12	
P. fuscovaginae NCPPB 3085^T	9	

*CFBP, Collection Franĉaise de Bactéries Phytopathogènes, Station de Pathologie Végétale et Phytobactériologie, Institut National de la Recherche Agronomique, Beaucouzé, France. For definitions of other abbreviations, see footnote to Table 1.

† RBR, Relative binding ratio.

Analysis of sequence data. The 16S rRNA gene sequence determined and the sequences of other pseudomonad reference strains obtained from the EMBL database were aligned and analysed using the CLUSTAL X program (Thompson *et al.*, 1997). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) and a phylogenetic tree was constructed by the neighbour-joining method of Saitou & Nei (1987). An evaluation of the tree was carried out using the bootstrap method (Felsenstein, 1985). A total of 1000 bootstrapped trees was generated. Calculations of levels of sequence similarity were based on data for 1322 nt.

RESULTS

Numerical analysis of phenotypic characters

Fifty of the 280 characters determined for all the strains of this study were either universally positive or

negative and were excluded from the analysis. At a similarity level of 62%, the numerical analysis delineated nine main clusters, (Fig. 1; Table 1). Cluster III could be further divided at a higher level of similarity into two subclusters IIIa and IIIb. Ten strains formed single strain clusters: *Pseudomonas caricapapayae* NCPPB 1873^T, *P. syringae* ATCC 19310^T, *Pseudomonas fragi* CCUG 1313^T, *P. cichorii* DSM 50259^T, *P. agarici* ATCC 25941^T, *Pseudomonas asplenii* ATCC 23835^T, *P. fluorescens* biovar V DSM 50148, *P. fluorescens* biovar II ATCC 17815, *P. putida* CFML 90-65 and *P. fluorescens* biovar IV DSM 50415. Phenotypic characteristics of the type strains of the reference species used in this study were in good agreement with published data (Barrett *et al.*, 1986; Coroler *et al.*, 1996; Dabboussi *et al.*, 1998; Elomari *et al.*, 1996; Grimont *et al.*, 1996; Palleroni, 1984; Stanier *et al.*, 1966): cluster II ($n = 5$) included *P. marginalis* ATCC 10844^T and two *P. marginalis* strains of plant origin and two *P. fluorescens* strains; subcluster IIIa ($n = 8$) contained the type strain *P. putida* biovar A ATCC 12633^T and seven *P. putida* strains isolated from clinical specimens; subcluster IIIb ($n = 5$) included the type strain *P. monteilii* CIP 104883^T and four strains of the same species isolated from human clinical specimens; cluster VII ($n = 2$) comprised the type strain of *P. aeruginosa* ATCC 10145^T and a strain of the same species from clinical origin; cluster IX ($n = 2$) contained the type strain of *Pseudomonas lundensis* CCM 573^T and the strain *P. lundensis* CCUG 18758, both

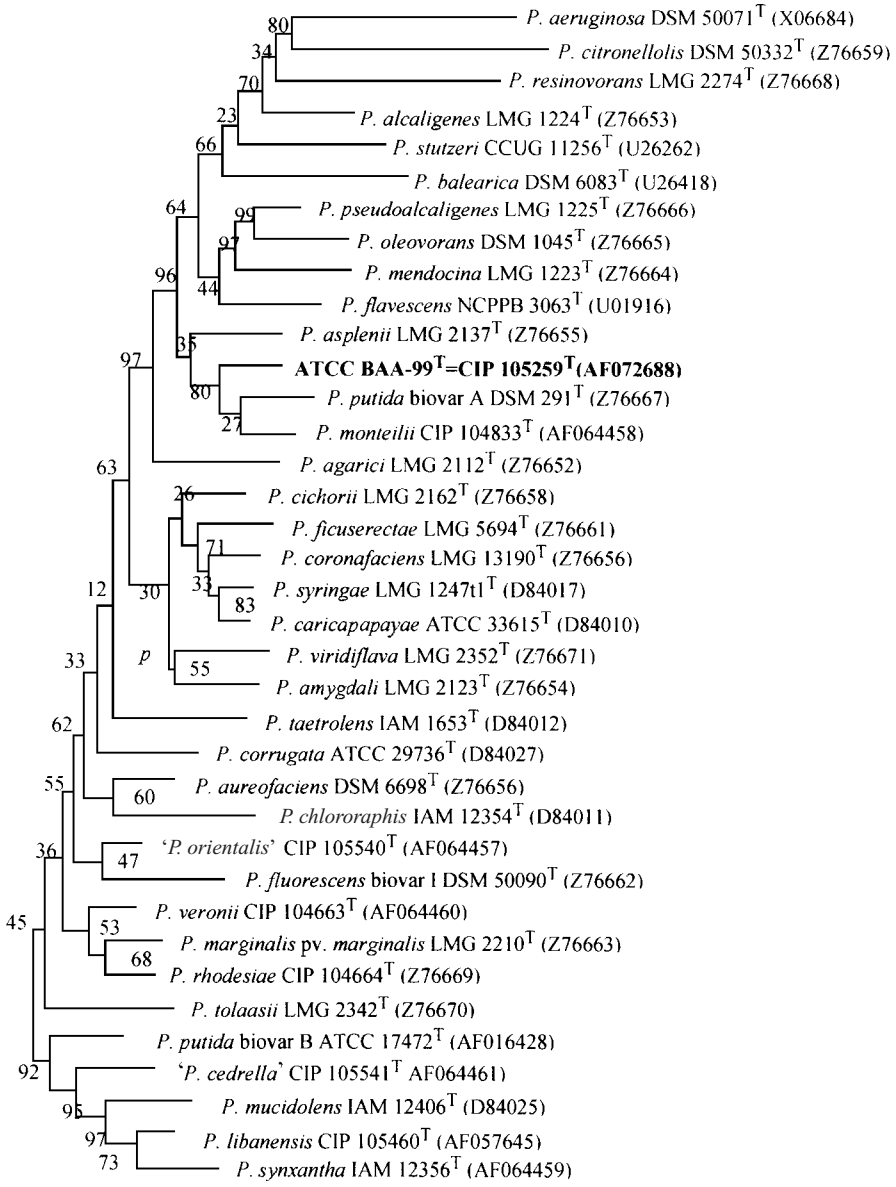

Fig. 2. Unrooted tree constructed using the neighbour-joining method showing the phylogenetic relationships of *P. mosselii* (= ATCC BAA-99^T = CIP 105259^T) and other species of the genus *Pseudomonas sensu stricto*. Numbers indicate the percentage occurrence of the branch in bootstrapped trees.

isolated from beef. The following clusters included two type strains of the reference species: cluster V ($n = 2$) grouped the type strains *Pseudomonas aureofaciens* ATCC 13985^T and *Pseudomonas chlororaphis* DSM 50083^T, which have been shown to be members of the same genospecies (Champion *et al.*, 1980); cluster I ($n = 4$) included two type strains *P. tolaasii* NCPPB 2192^T and *P. fluorescens* biovar I ATCC 13525^T, associated with *P. tolaasii* strain NCPPB 1616 and *P. fluorescens* biovar I strain ATCC 17563.

The 22 strains of our collection isolated from clinical specimens were distributed as follows: 1 strain (*P. fluorescens*) in cluster II, 8 strains (*P. putida*) in cluster III, 12 strains (1 *P. putida*, 2 *Pseudomonas* sp., 9 *P. fluorescens*) in cluster IV and 1 strain (*P. putida*) remained unclustered. This last cluster (cluster IV) did not include any type strain or strains from any other collection (Table 1). The phenotypic data of the 12 strains of cluster IV (proposed below as *P. mosselii* sp. nov.) are compared in Table 2 with those of other species, among them the non-phytopathogenic members of section I pseudomonads (Palleroni *et al.*, 1973): *P. aeruginosa*, *P. fluorescens* biovars I–V, *P. putida* biovar A and B and *P. chlororaphis* and the newly described species *P. veronii* (Elomari *et al.*, 1996), *P. rhodesiae* (Coroler *et al.*, 1996), *P. monteilii* (Elomari *et al.*, 1997), '*P. cedrella*', '*P. orientalis*' (Dabboussi *et al.*, 1999a) and *P. libanensis* (Dabboussi *et al.*, 1999b).

DNA–DNA hybridization and thermal stability

Results of DNA–DNA hybridizations obtained with labelled DNA of strain CFML 90-83^T are shown in Table 3. Hybridization values between strains within cluster IV were 72–100 % and the ΔT_m value was 1–4 °C. Hybridization experiments were also performed between strain CFML 90-83^T and the 10 clinical isolates belonging to the other clusters identified by phenotypic analysis. All values were in the range 10–41 %. The highest DNA–DNA hybridization value was obtained with *P. monteilii* CIP 104833^T (49 %), with a ΔT_m of 10 °C. Table 3 also shows the level of reassociation between strain CFML 90-83^T and all of the reference strains of the genus *Pseudomonas* used in this study (67 strains). All values were less than 49 %, with ΔT_m values of 8–10 °C.

DNA base composition

The G+C contents of strains CFML 90-83^T, CFML 90-74, CFML 90-70 and CFML 90-80 were 63, 62, 61 and 62 mol %, respectively.

16S rDNA sequence analysis

The sequence of 1322 nt of the 16S rDNA gene of strain CFML 90-83^T was aligned to database-available sequences of other species of the genus *Pseudomonas sensu stricto* (Kersters *et al.*, 1996), including six newly described species: *P. veronii* (Elomari *et al.*, 1996), *P. rhodesiae* (Coroler *et al.*, 1996), *P. monteilii* (Elomari *et al.*, 1997), *P. libanensis* (Dabboussi *et al.*, 1999b), '*P. cedrella*' and *P. orientalis* (Dabboussi *et al.*, 1999a). The tree constructed by the neighbour-joining method (Saitou & Nei, 1987) and showing phylogenetic relationships of strain CFML 90-83^T with 36 type strains is illustrated in Fig. 2. Its closest relatives are *P. monteilii*, *P. putida* biovar A and *P. asplenii*. Levels of nucleotide similarities for the 16S rDNA sequence of strain CFML 90-83^T and other *Pseudomonas* species were greater than 95·3 % (*Pseudomonas resinovorans*). Strain CFML 90-83^T was most closely related, in terms of sequence similarity, to *P. monteilii* (99·4 %). Our 16S rDNA sequence comparison confirmed that strain CFML 90-83^T belongs to the genus *Pseudomonas*.

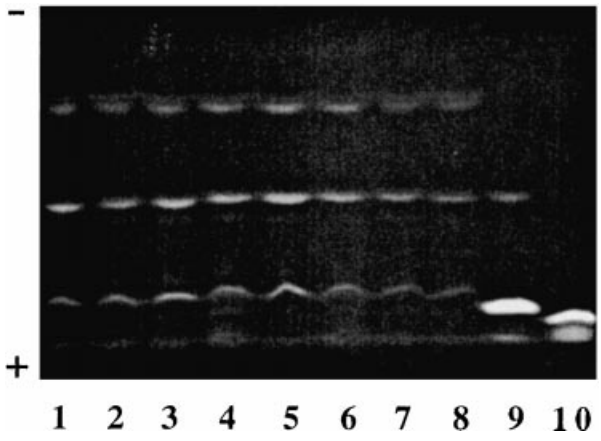

Fig. 3. IEF of the pyoverdines produced by *P. mosselii* strains CFML 90-71, 90-73, 90-74, 90-77, 90-78, 90-80, 90-82, 90-83^T (lanes 1–8, respectively), *P. monteilii* CFML 90-54 (lane 9) and *P. putida* ATCC 12633^T (lane 10).

Characterization of strains by pyoverdine typing

The pyoverdines synthesized during growth under iron starvation conditions by isolates of phenotypic cluster IV all presented an identical IEF pattern (Fig. 3) for 8 different isolates. Three major pyoverdine bands, corresponding to different isoforms of an otherwise identical molecule (Budzikiewicz, 1997), were characterized by pH_i values of 9·0, 7·4 and 5·2, respectively (from top to bottom in Fig. 3, lanes 1–8). Several supplementary minor bands were more or less visible, depending on the amount of pyoverdines accumulated by the strains during growth. None of the 37 strains analysed among the other fluorescent pseudomonad species listed in Table 3 displayed the same IEF profile, as illustrated for two strains, *P. monteilii* CFML 90-54 (Fig. 3, lane 9) and *P. putida* ATCC 12633^T (Fig. 3, lane 10). Strain CFML 90-75, unique among isolates of cluster IV, revealed no pyoverdine production when grown under iron-deficient conditions and, thus, remained uncharacterized by the IEF method. Therefore, the strain was tested for pyoverdine-mediated iron incorporation (Meyer *et al.*, 1997) and demonstrated an efficient capacity, identical to that of the

Table 4. Variable characters within the 12 *P. mosselii* strains

+, Positive; −, negative; C, 50 % of strains positive. All type strain reactions were identical to those of the majority of *P. mosselii* strains, except for: gelatinase, Tween esterase, fibrinolysis, assimilation of L-threonine, ethanolamine, levulinate, adipate, 2-ketoglutarate, L-tyrosine arylamidase, L-ornithine arylamidase and *N*-acetyl-β-D-glucosaminidase. All *P. mosselii* strains utilized the following substrates as carbon and energy sources: glycerol, ribose, D-glucose, D-fructose, *N*-acetyl-glucosamine, gluconate, 2-ketogluconate, D-alanine, L-alanine, L-leucine, L-isoleucine, L-valine, L-serine, L-phenylalanine, L-histidine, L-aspartate, L-glutamate, L-ornithine, L-lysine, L-citrulline, L-arginine, betaine, β-alanine, DL-4-amino-butyrate, DL-5-amino-valerate, sarcosine, diaminobutane, spermine, histamine, acetate, propionate, butyrate, isobutyrate, n-valerate, isovalerate, n-caproate, heptanoate, caprylate, pelargonate, caprate, glutarate, DL-lactate, DL-glycerate, DL-3-hydroxy-butyrate, D-malate, *meso*-tartrate, pyruvate, aconitate, citrate, phenyl-acetate, *p*-hydroxy-benzoate, L-proline, succinate, fumarate and L-malate. No strain used the following substrates as carbon and energy sources: erythritol, D-arabinose, L-arabinose, D-xylose, adonitol, methyl β-xyloside, L-sorbose, dulcitol, inositol, methyl α-D-glucoside, amygdalin, arbutin, glycogen, β-gentiobiose, D-lyxose, L-arabitol, 5-ketogluconate, L-norleucine, DL-norvaline, DL-2-aminobutyrate, L-tryptophan, DL-kynurenine, creatine, DL-3-amino-butyrate, DL-2-amino-benzoate, acetamide, benzylamine, tryptamine, malonate, maleate, pimelate, suberate, azelate, sebacate, glycolate, D-tartrate, L-tartrate, citraconate, itaconate, mesaconate, benzoate, *o*-hydroxy-benzoate, *m*-hydroxy-benzoate, D-mandelate, L-mandelate, phthalate, tere-phthalate, L-xylose, methyl α-D-mannoside, salicin, lactose, D-melebiose, inulin, D-tagatose, D-fucose, L-cysteine, D-tryptophan, DL-3-amino-benzoate, DL-4-amino-benzoate, ethylamine, oxalate, iso-phthalate. All strains possess the following enzyme activities: L-hydroxyproline arylamidase, L-histidine arylamidase, glycine arylamidase, L-arginine arylamidase, γ-glutamyltransferase, methionine arylamidase, glycyl-glycine arylamidase, glycyl-phenylalanine arylamidase, leucyl-glycine arylamidase, L-glutamine arylamidase, L-proline arylamidase, L-serine arylamidase, β-alanine arylamidase, glycyl-L-alanine arylamidase, glycyl-L-tryptophan arylamidase, L-leucyl-L-alanine arylamidase, L-seryl-L-methionine arylamidase, L-lysine arylamidase, L-alanine arylamidase, and esterases-C4, -C6, -C8, -C9, -C10 and -C19. No strains possess the following enzyme activities: pyrrolidone arylamidase, L-aspartate arylamidase, *N*-CBZ-glycyl-arginine arylamidase, L-alanyl-L-phenylalanyl-L-proline arylamidase, L-alanyl-L-phenylalanyl-L-prolyl-L-alanine arylamidase, α-L-aspartyl-L-alanine arylamidase, α-L-aspartyl-L-arginine arylamidase, *N*-benzoyl-leucine arylamidase, *S*-benzoyl-cysteine arylamidase, *N*-CBZ-arginine-4-methoxy arylamidase, L-leucyl-L-leucyl-L-valyl-L-tyrosyl-L-serine arylamidase, *N*-benzoyl-L-alanyl-4-methoxy arylamidase, *N*-acetyl-glycyl-L-lysine arylamidase, α-D-galactosidase, β-D-galactosidase, phospho-β-D-galactosidase, α-L-arabinosidase, β-D-galacturonohydrolase, β-D-glucuronidase, β-maltosidase, *N*-acetyl-α-D-glucosaminidase, α-D-fucosidase, β-L-fucosidase, β-D-lactosidase, α-D-mannosidase, β-D-mannosidase, α-D-xylosidase, β-D-xylosidase, α-D-glucosidase, β-D-glucosidase, α-maltosidase, α-L-fucosidase, esterases-C16, L-lysyl-L-serine-4-methoxy arylamidase.

Character	*P. mosselii* strains ($n = 12$)*	Type strain CFML 90-83^T
Conventional tests:		
Gelatinase	+ (11)	−
Tween esterase	C (6)	−
Lecithinase, haemolysis	− (8)	−
Fibrinolysis	+ (8)	−
Carbon sources:		
Galactose, rhamnose, maltose, sucrose, xylitol, glucosamine	− (10)	−
Butylamine, amylamine	+ (11)	+
Mannitol, D-arabitol, L-tyrosine	+ (9)	+
Sorbitol, aesculin, D-melezitose, D-turanose, L-fucose, D-raffinose	− (10)	−
D-Cellobiose	− (8)	−
Glycine, L-methionine	− (7)	−
Starch	− (11)	−
L-Threonine	+ (9)	−
Ethanolamine, levulinate	+ (11)	−
Adipate	− (11)	+
Enzymic tests:		
L-Tyrosine arylamidase	− (7)	+
L-Phenylalanine arylamidase, L-tryptophan arylamidase	+ (10)	+
Glycyl-proline arylamidase	− (7)	−
L-Seryl-tyrosine arylamidase, L-alanyl-L-arginine arylamidase, glycyl-L-arginine arylamidase, L-phenylalanyl-L-arginine arylamidase, L-phenylalanyl-L-proline arylamidase, L-threonine arylamidase, L-arginyl-L-arginine arylamidase, L-histidyl-L-leucyl-L-histidine arylamidase	− (10)	−
α-L-Glutamate arylamidase, L-isoleucine arylamidase, α-L-glutamyl-L-histidine arylamidase, L-histidyl-L-serine arylamidase, L-phenylalanyl-L-prolyl-L-alanine arylamidase	− (11)	−

Table 4 (*cont.*)

Character	*P. mosselii* strains ($n = 12$)*	Type strain CFML 90-83^T
L-Ornithine arylamidase,	+ (7)	−
L-Lysyl-L-alanine arylamidase	+ (11)	+
L-Lysyl-L-lysine arylamidase, L-valyl-L-tyrosyl-L-serine arylamidase	−(9)	−
L-Histidyl-L-phenylalanine arylamidase	− (8)	−
N-Acetyl-*β*-D-glucosaminidase	+ (10)	−
Esterases-C5, -C12, -C14	+ (11)	+

* Numbers in parentheses are the number of strains with the recorded result.

other isolates of cluster IV, for incorporating iron liganded to the pyoverdine of strain CFML 90-83^T (data not shown).

DISCUSSION

Twenty-two strains isolated from clinical specimens received as *P. putida* (10 strains), *P. fluorescens* (10 strains) and *Pseudomonas* sp. (2 strains) were studied by a polyphasic taxonomic approach, including 16S rDNA phylogeny, numerical analysis, DNA–DNA hybridization and thermal stability of DNA–DNA hybrids, as well as by the recently described siderotyping methodology (Meyer *et al.*, 1997). These strains, which phenotypically belong to the fluorescent pseudomonad group, were mainly grouped (19 strains) into two phenotypic clusters, IIIa and IV. Phenotypic cluster IIIa also included the *P. putida* biovar A type strain. Cluster IV contained 12 clinical isolates, but no type strains or strains from other collections. These results suggest that cluster IV isolates belong to the genus *Pseudomonas sensu stricto*. To further unravel the finer taxonomic position of the cluster IV isolates within this genus, DNA–DNA hybridizations were performed within this cluster and with the type and other representative strains of related groups or species. Cluster IV constituted a separate DNA hybridization group (72–100% hybridization, ΔT_m values less than 4 °C) with only low levels of DNA hybridization with other species of the genus *Pseudomonas sensu stricto*, including the newly described species *P. veronii*, *P. rhodesiae*, *P. monteilii*, *P. libanensis*, '*P. cedrella*' and '*P. orientalis*'. Furthermore, the DNA–DNA hybridization values obtained between strain CFML 90-83^T and all strains of cluster IIIa were low. The ΔT_m values obtained for hybrids between strain CFML 90-83^T and reference strains of the genus *Pseudomonas* were high (9–10 °C). At present, the species is the basic unit of bacterial taxonomy and is defined as a group of strains sharing 70% or greater DNA–DNA relatedness with a ΔT_m value of 5 °C or less. Both values have to be considered and phenotypic features also have to agree with this definition (Stackebrandt & Goebel, 1994).

The mean DNA G+C content was 62 mol% for the four strains studied (CFML 90-83^T, CFML 90-74, CFML 90-70 and CFML 90-80). This value is typical of the genus *Pseudomonas sensu stricto* (58–70 mol%; Palleroni, 1984). The derived 16S rRNA sequence of strain CFML 90-83^T was aligned and compared with the sequences of other *Pseudomonas* species retrieved from the EMBL database. Strain CFML 90-83^T exhibited the lowest level of 16S rRNA sequence similarity with *P. resinovorans* (95·3%). The highest level of sequence relatedness was observed with *P. monteilii* CIP 104883^T (99·4%), but strains of cluster IV could be differentiated from *P. monteilii* by DNA–DNA hybridization and several phenotypic features such as assimilation of D-mannose, *meso*-tartrate, *myo*-inositol and benzoate. Furthermore, the phylogenetic tree shows that the node of the *P. mosselii*/*P. putida* biovar A/*P. monteilii* grouping appeared with an occurrence of 80%, which is considered to be high. In fact, molecular methods are essential for determining the precise taxonomic organization of the bacterial world. Comparisons of rRNA sequences of corresponding genes allow phylogenetic reconstruction (De Vos & De Ley, 1983; Fox *et al.*, 1980; Grimont & Grimont, 1986; Palleroni *et al.*, 1973). The taxonomic information thus obtained is invaluable above the species level and for species delineation; however, quantitative DNA–DNA hybridization presently remains the most straightforward method for describing a species (Wayne *et al.*, 1987). The phylogenetic tree shows that strain CFML 90-83^T falls within the genus *Pseudomonas* and specifically within the *P. fluorescens* intrageneric cluster as defined by Moore *et al.* (1996). The pyoverdine typing performed by IEF was also a powerful molecular method for discriminating the members of cluster IV from all other fluorescent *Pseudomonas* species so far analysed. An identical pyoverdine IEF pattern suggests production of a structurally identical pyoverdine that appeared to be specific to isolates of cluster IV. Initially developed for the differentiation of *P. aeruginosa* isolates (Meyer *et al.*, 1997) and also successfully used recently for recognition of the novel species *Pseudomonas brassicacearum* and *Pseudomonas thivervalensis* (Achouak *et al.*, 2000), siderotyping appears to be a promising new methodology for taxonomic purposes among the fluorescent *Pseudomonas* spp. Furthermore, pyoverdine-mediated iron uptake studies could replace

pyoverdine IEF analysis for strains naturally deficient in pyoverdine production, as shown in the present study for *P. mosselii* CFML 90-75.

As a conclusion of the polyphasic study, a novel *Pseudomonas* species, *P. mosselii* sp. nov., is proposed for the 12 strains of cluster IV. Affiliation to the genus *Pseudomonas* is justified by the close phylogenetic relationships of strain CFML 90-83^T to other strains representative of the genus. The low level of DNA–DNA relatedness to other *Pseudomonas* strains justifies the creation of *P. mosselii* as a novel species. *P. mosselii* strains are phenotypically and genotypically (DNA–DNA hybridization) homogeneous, with a clear and characteristic set of phenotypic features (Table 2 and Table 4) that allow identification to the species level. *P. mosselii* strains could be differentiated from related fluorescent members of the genus *Pseudomonas* by several phenotypic features (Table 2). Differentiation of *P. mosselii* and *P. aeruginosa* is based on pyocyanin production, denitrification, growth at 41 °C and assimilation of D-mannose, malonate, *meso*-tartrate, benzoate and α-aminobutyrate. *P. fluorescens* (all biovars) and *P. putida* biovar A could be generally differentiated from *P. mosselii* by the assimilation of α-aminobutyrate. *P. mosselii* and *P. putida* biovar B differed phenotypically since all strains of *P. putida* biovar B assimilate mucate, malonate, benzoate, tryptamine and L-tryptophan, whereas strains of *P. mosselii* are unable to utilize these compounds.

Moreover, *P. mosselii* strains could be characterized by their unique pyoverdine IEF pattern. Investigations of a larger collection of bacteria is in progress to assess the use of this method for taxonomic purposes among the fluorescent *Pseudomonas*.

Description of *Pseudomonas mosselii* sp. nov.

Pseudomonas mosselii (mos.se′li.i. M.L. masc. gen. n. *mosselii* of Mossel, in honour of David A. A. Mossel, a Dutch microbiologist, for his contribution to medical and food microbiology).

Sources of the *P. mosselii* strains are shown in Table 1. Gram-negative, motile by a single polar flagellum, asporogenous and rod-shaped. Cells produce a fluorescent pigment on King B medium, but do not produce phenazine pigments on King A medium. Colonies on nutrient agar are circular and non-pigmented. Temperature range for growth is 10–36 °C; optimal growth occurs at 30 °C. Growth occurs in the presence of 3 and 5% NaCl, but not in the presence of 7% NaCl. Able to grow on cetrimide. Non-haemolytic on blood agar. Does not reduce nitrate to nitrite. Negative for elastase, coagulase, chondroitinase, hyaluronidase and tetrathionate reductase. Arginine dihydrolase, catalase and cytochrome oxidase are produced. Lysine and ornithine are not decarboxylated. L-Tyrosine is hydrolysed, but not aesculin. Negative Voges–Proskauer reaction and tributyrin test. No action against DNA or RNA. Phenotypic characters which are either positive or negative for all the *P. mosselii* strains are listed in Table 4. Variable characteristics of 12 *P. mosselii* strains are shown in Table 4. G+C content of the type strain is 63 mol%. All strains were isolated from clinical specimens, but the clinical significance of *P. mosselii* is not known. Type strain is CFML 90-83^T (= ATCC BAA-99^T = CIP 105259^T).

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