***Pseudomonas libanensis* sp. nov., a new species isolated from Lebanese spring waters**

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The taxonomic position of eight fluorescent *Pseudomonas* isolates, from two Lebanese spring waters, which were previously recognized by numerical analysis as members of a new subcluster (subcluster Vb) was examined. Except for one strain, the new subcluster exhibited internal DNA hybridization values of 76–100%, and 9–53% hybridization was measured with the type or reference strains of other *Pseudomonas* species. The highest DNA binding value was found with *Pseudomonas marginalis* strains (37–53%). The G+C content of the DNA of the type strain was 58 mol%. A comparison of 1322 nt of the 16S rRNA gene sequence of the strain representing subcluster Vb (CFML 96-195^T) with the sequence of other strains of the genus *Pseudomonas* revealed that strain CFML 96-195^T was part of the '*Pseudomonas fluorescens* intragenetic cluster'. On the basis of the results of phenotypic, DNA–DNA and phylogenetic analyses, a new *Pseudomonas* species, *Pseudomonas libanensis* sp. nov., is proposed for the seven strains of subcluster Vb. The type strain is *P. libanensis* CFML 96-195^T and has been deposited in the Collection de l'Institut Pasteur (Paris, France) as CIP 105460^T. The *P. libanensis* strains are phenotypically and genotypically homogeneous and can be differentiated from most other fluorescent species by several phenotypic features. Differentiation of *P. libanensis* and *Pseudomonas aeruginosa* is based mainly on pyocyanin production; *P. libanensis* can be differentiated from *P. fluorescens* (all biovars) by α -aminobutyrate assimilation. The clinical significance of *P. libanensis* is unknown.

Keywords: *Pseudomonas libanensis* sp. nov., DNA–DNA hybridization, 16S rRNA

INTRODUCTION

The aerobic pseudomonads are bacteria of considerable scientific and practical importance; they are widespread in nature and are being isolated in increasing numbers and kind. Fluorescent *Pseudomonas* strains constitute a diverse group of bacteria that can generally be visually distinguished from other pseudomonads by their ability to produce a water-soluble yellow-green pigment. On the basis of DNA–rRNA hybridization, Palleroni *et al.* (1973) have enabled subdivision of the genus *Pseudomonas* into five homology groups. Sequence analysis of the 16S rRNA gene has provided a clear framework for the systematic grouping of the five homology groups. It is now

generally recognized that the genus *Pseudomonas sensu stricto* should be limited to those organisms clustering in DNA–rRNA homology group I (De Vos & De Ley, 1983) in the γ -subclass of the *Proteobacteria* (Woese, 1987). Many organisms originally described as species of the genus *Pseudomonas* have been reclassified in other genera and families (De Vos *et al.*, 1985; Gillis *et al.*, 1995; Palleroni & Bradbury, 1993; Segers *et al.*, 1994; Swings *et al.*, 1983; Urakami *et al.*, 1994; Willems *et al.*, 1989, 1990, 1991, 1992; Yabuuchi *et al.*, 1992, 1995). The genus *Pseudomonas sensu stricto* includes both fluorescent and non-fluorescent species (*Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes* and *Pseudomonas fragi*). The saprophytic fluorescent pseudomonads are characterized by the production of water-soluble pigments (pyoverdins) and can be distinguished from phytopathogenic species by their positive arginine dihy-

The EMBL accession number for the 16S rRNA gene sequence of strain CFML 96-195^T is AF057645.

Table 1. Levels of DNA–DNA hybridization of labelled strain CFML 96-195^T with phenotypic clusters or subclusters

CFML, Collection de la Faculté de Médecine de Lille, Lille, France; NC, strain not belonging to any cluster. All strains were isolated from three Lebanese spring waters, A, B and C (Kadicha, Kassam and Mar-Sarkis, respectively) at the point of emergence. RBR, Relative binding ratio of DNAs.

Phenotypic cluster or subcluster	Strain (CFML no.)	Origin of spring water	Labelled DNA from strain CFML 96-195 ^T		G + C content (mol%)
			RBR (%)	ΔT_m (°C)	
Vb	96-195 ^T	A	100	0	58
	96-172	C	95		
	96-194	A	95		59
	96-193	A	94		
	96-199	A	85		
	96-192	A	84	1	
	96-183	A	76	1	58
	96-210	A	61	8	
Ia	96-217	A	30		
	96-197	A	43		
II	96-171	C	46		
	96-201	A	36		
	96-169	B	43		
	96-219	A	40		
	96-184	A	42		
	96-216	A	48		
	96-190	A	40		
	96-200	A	52	8	
IVa	96-196	A	48		
	96-186	A	47		
Va	96-205	A	46		
	96-175	C	40		
	96-173	C	41		
Vc	96-180	A	45		
	96-211	A	47		
	96-170	B	61	8	
Vd	96-164	B	50	9	
	96-167	B	56	9	
	96-178	C	54	9	
	96-166	B	50	9	
	96-181	A	41		
	96-188	A	67	7	
	96-179	C	49		
	96-215	A	51	9	
Ve	96-209	A	53	9	
	96-176	C	52	9	
	96-214	A	60	8	
	96-213	A	58	9	
	96-174	C	54	9	
	96-163	B	41		
	96-198	A	56	9	
	96-206	A	43		
Vf	96-189	A	41		
	96-204	A	44		
	96-177	C	48		
	96-207	A	39		
	96-185	A	43		
	96-191	A	39		
	96-182	A	37		

Table 1. (cont.)

Phenotypic cluster or subcluster	Strain (CFML no.)	Origin of spring water	Labelled DNA from strain CFML 96-195 ^T		G + C content (mol %)
			RBR (%)	ΔT_m (°C)	
Vg	96-212	A	47		
	96-203	A	43		
VI	96-187	A	46		
	96-218	A	53	8	
	96-208	A	44		
	96-165	B	35		
VIIIa	96-202	A	40		
	96-162	B	18		
NC	96-168	B	24		

drolase reaction. The complexity of fluorescent saprophyte species other than *Pseudomonas aeruginosa* (considered a homogeneous species; Palleroni, 1984) has been well illustrated by extensive studies (Barrett *et al.*, 1986; Champion *et al.*, 1980; Molin & Ternström 1982; Palleroni *et al.*, 1973; Elomari *et al.*, 1997).

Nevertheless, pseudomonad identification at the species level continues to be a difficult task, especially in environmental studies where this group is a majority, such as aquatic ecosystems. Natural waters can be characterized by their bacterial flora which is considered to be an indicator of the quality of the water. Leclerc & Guillot (1992) showed that approximately 80% of strains isolated from natural waters were not identifiable at the species level and that the majority of strains which could be identified were fluorescent members of the genus *Pseudomonas*. These results have revealed the need to greatly improve our understanding of fluorescent pseudomonads in the natural environment.

In a previous study (Dabboussi *et al.*, 1998) we performed a numerical analysis with 58 isolates. These were isolated at the point of emergence of three important spring waters in North Lebanon that feed 45% of the Lebanese population (Kadicha, Kassam and Mar-Sarkis). These isolates were identified as fluorescent pseudomonads. This numerical analysis indicated the presence of three phenotypic subclusters (Vb, Vd and Ve), including strains found only in Lebanese spring water. This paper describes the phenotypic, genotypic (DNA–DNA hybridization, ΔT_m , G+C content) and phylogenetic (16S rDNA sequence analysis) properties of subcluster Vb (Dabboussi *et al.*, 1998) and proposes a new species, *Pseudomonas libanensis* sp. nov., for seven strains of this subcluster. The type strain (CFML 96-195^T) has been deposited in the Collection de l'Institut Pasteur (Paris, France) as CIP 105460^T.

METHODS

Bacterial strains. A total of 121 strains were used in this study: 58 wild strains, previously listed in detail (Dabboussi *et al.*, 1998), isolated from three Lebanese spring waters (Table 1) and 65 reference strains used in DNA–DNA hybridizations and representing 24 known species of the genus *Pseudomonas sensu stricto* (Kerstens *et al.*, 1996) and three newly described *Pseudomonas* species, *Pseudomonas veronii* (Elomari *et al.*, 1996), *Pseudomonas rhodesiae* (Coroller *et al.*, 1996), both isolated from French mineral waters, and *Pseudomonas monteilii* (Elomari *et al.*, 1997) isolated from clinical specimens (Table 2). All bacteria were cultured routinely on Mueller–Hinton medium at 30 °C.

Biochemical, physiological and flagellar characteristics. Phenotypic data for the 121 strains used in this study have been described previously (Dabboussi *et al.*, 1998). The flagellation of the bacteria was investigated by electron microscopy using a negative-staining technique (Hoeniger, 1965) on fixed organisms. The stained bacteria were examined with a JEOL type 100 CX transmission electron microscope.

DNA extraction and purification. Chromosomal DNA was isolated and purified according to the method of Beji *et al.* (1987).

DNA base composition. The G+C content of DNA was determined from the mid-point value of the thermal denaturation profile. The G+C content was calculated by using the equation of De Ley (1970) and *Escherichia coli* ATCC 11775^T DNA was used as reference (G+C content 51 mol%).

DNA–DNA similarity. DNA–DNA hybridization tests were carried out by using labelled DNAs from strain CFML 96-195^T (subcluster Vb; Dabboussi *et al.*, 1998). Native DNA was labelled *in vitro* by nick translation with tritium-labelled nucleotides. The S1 nuclease–trichloroacetic acid method used for hybridization has been described by Crosa *et al.* (1973) and Grimont *et al.* (1980). The reaction mixture of radioactively labelled DNA and unlabelled DNA was incubated for 16 h at 60 °C.

Table 2. Levels of DNA relatedness of *P. libanensis* to different type and collection strains of the genus *Pseudomonas*

RBR, Relative binding ratio of DNAs.

Source of unlabelled DNA*	Labelled DNA from strain CFML 96-195 ^T		Source of unlabelled DNA*	Labelled DNA from strain CFML 96-195 ^T	
	RBR (%)	ΔT_m (°C)		RBR (%)	ΔT_m (°C)
<i>P. aeruginosa</i> ATCC 10145 ^T	15		<i>P. mucidolens</i> CIP 103298 ^T	42	
<i>P. aeruginosa</i> ATCC 27853	25				
<i>P. aeruginosa</i> ATCC 15692	20		<i>P. lundensis</i> CCM 573 ^T	14	
			<i>P. lundensis</i> CCUG 18785	20	
<i>P. fluorescens</i> biovar I ATCC 13525 ^T	50	8			
<i>P. fluorescens</i> biovar I ATCC 17563	51	6	<i>P. syringae</i> ATCC 19310 ^T	15	
<i>P. fluorescens</i> biovar II ATCC 17816	43				
<i>P. fluorescens</i> biovar II ATCC 17815	41		<i>P. savastanoi</i> CFBP 1670 ^T	22	
<i>P. fluorescens</i> biovar II ATCC 17482	44		<i>P. savastanoi</i> CFBP 2088	26	
<i>P. fluorescens</i> biovar II DSM 50106	38		<i>P. savastanoi</i> CFBP 1838	17	
<i>P. fluorescens</i> biovar III ATCC 17559	38				
<i>P. fluorescens</i> biovar III ATCC 17400	40		<i>P. viridiflava</i> ATCC 13223 ^T	21	
<i>P. fluorescens</i> biovar III ATCC 17571	43				
<i>P. fluorescens</i> biovar IV DSM 50415	35		<i>P. cichorii</i> DSM 50259 ^T	14	
<i>P. fluorescens</i> biovar IV ATCC 12983	30				
<i>P. fluorescens</i> biovar V ATCC 14150	45		<i>P. agarici</i> ATCC 25941 ^T	28	
<i>P. fluorescens</i> biovar V ATCC 17518	50	7			
<i>P. fluorescens</i> biovar V ATCC 15916	51	6	<i>P. asplenii</i> ATCC 23835 ^T	25	
<i>P. fluorescens</i> biovar V ATCC 17386	34				
<i>P. fluorescens</i> biovar V ATCC 17573	23		<i>P. caricapapayae</i> NCPPB 1873 ^T	17	
<i>P. fluorescens</i> biovar V DSM 50148	25				
			<i>P. tolaasii</i> NCPPB 2129 ^T	23	
<i>P. marginalis</i> ATCC 10844 ^T	40		<i>P. tolaasii</i> NCPPB 1616	43	
<i>P. marginalis</i> DSM 50275	53	8			
<i>P. marginalis</i> DSM 50276	37		<i>P. stutzeri</i> ATCC 17588 ^T	33	
			<i>P. stutzeri</i> ATCC 17591	11	
<i>P. chlororaphis</i> DSM 50083 ^T	25		<i>P. stutzeri</i> ATCC 17587	20	
<i>P. chlororaphis</i> ATCC 9447	35		<i>P. stutzeri</i> ATCC 17686	16	
<i>P. chlororaphis</i> ATCC 17414	35				
			<i>P. mendocina</i> ATCC 25411 ^T	13	
<i>P. aureofaciens</i> CCEB 518 ^T	45		<i>P. mendocina</i> ATCC 25412	18	
<i>P. aureofaciens</i> ATCC 17415	40				
			<i>P. alcaligenes</i> ATCC 14909 ^T	14	
<i>P. veronii</i> CIP 104663 ^T	42				
			<i>P. pseudoalcaligenes</i> ATCC 17440 ^T	15	
<i>P. rhodesiae</i> CIP 104664 ^T	43		<i>P. pseudoalcaligenes</i> ATCC 12815	18	
<i>P. putida</i> biovar A ATCC 12633 ^T	16		<i>P. fragi</i> ATCC 4973 ^T	22	
<i>P. putida</i> biovar A DSM 50208	26		<i>P. fragi</i> ATCC 27362	26	
<i>P. putida</i> biovar B ATCC 17484	31				
<i>P. putida</i> biovar B ATCC 17430	23		<i>P. flavescens</i> NCPPB 3063 ^T	25	
<i>P. putida</i> biovar B CCUG 1317	25		<i>P. flavescens</i> CIP 104205	19	
<i>P. monteilii</i> CIP 104883 ^T	9		<i>P. fuscovaginae</i> NCPPB 3085 ^T	23	
<i>P. synxantha</i> CIP 5922 ^T	49				

* ATCC, American Type Culture Collection, Manassas, VA, USA; CCEB, Culture Collection of Entomogenous Bacteria, Institute of Entomology, Czechoslovakia Academy of Sciences, Prague, Czech Republic; CCM, Czechoslovak Collection of Microorganisms, F. E. Purkyne University of Brno, Brno, Czech Republic; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CFBP, Collection Française de Bactéries Phytopathogènes, Station de Pathologie Végétale et Phytobactériologie, Institut National de la Recherche Agronomique, Beaucauzé, France; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCIB, National Collection of Industrial Bacteria, Aberdeen, UK; NCPPB, National Collection of Plant-Pathogenic Bacteria, Plant Pathology Laboratory, Hatching Green, Harpenden, UK.

Table 3. Strains used for the 16S rDNA sequence analysis

Species	Strain*	EMBL accession no.
<i>P. libanensis</i>	CIP 105460 ^T	AF057645
<i>P. aeruginosa</i>	DSM 50071 ^T	X06684
<i>P. agarici</i>	LMG 2112 ^T	Z76652
<i>P. alcaligenes</i>	LMG 1224 ^T	Z76653
<i>P. amygdali</i>	LMG 2123 ^T	Z76654
<i>P. asplenii</i>	LMG 2137 ^T	Z76655
<i>P. aureofaciens</i>	DSM 6698 ^T	Z76656
<i>P. balearica</i>	DSM 6083 ^T	U26418
<i>P. caricapapayae</i>	ATCC 33615 ^T	D84010
<i>P. chlororaphis</i>	IAM 12354 ^T	D84011
<i>P. cichorii</i>	LMG 2162 ^T	Z76658
<i>P. citronellolis</i>	DSM 50332 ^T	Z76659
<i>P. coronafaciens</i>	LMG 13190 ^T	Z76660
<i>P. corrugata</i>	ATCC 29736 ^T	D84027
<i>P. ficuserectae</i>	LMG 5694 ^T	Z76661
<i>P. flavesceus</i>	NCPBB 3063 ^T	U01916
<i>P. fluorescens</i> biovar I	DSM 50090 ^T	Z76662
<i>P. marginalis</i> pv. <i>marginalis</i>	LMG 2210 ^T	Z76663
<i>P. mendocina</i>	LMG 1223 ^T	Z76664
<i>P. monteilii</i>	CIP 104883 ^T	AF064458
<i>P. mucidolens</i>	IAM 12406 ^T	D84025
<i>P. oleovorans</i>	DSM 1045 ^T	Z76665
<i>P. putida</i> biovar A	DSM 291 ^T	Z76667
<i>P. putida</i> biovar B	ATCC 17472	AF016428
<i>P. pseudoalcaligenes</i>	LMG 1225 ^T	Z76666
<i>P. resinovorans</i>	LMG 2274 ^T	Z76668
<i>P. rhodesiae</i>	CIP 104664 ^T	Z76669
<i>P. stutzeri</i>	CCUG 11256 ^T	U26262
<i>P. synxantha</i>	IAM 12356 ^T	AF064459
<i>P. syringae</i>	LMG 1247t1 ^T	D84017
<i>P. taetrolens</i>	IAM 1653 ^T	D84012
<i>P. tolaasii</i>	LMG 2342 ^T	Z76670
<i>P. veronii</i>	CIP 104663 ^T	AF064460
<i>P. viridiflava</i>	LMG 2352 ^T	Z76671

* IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; LMG, Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Gent, Belgium; CIP, DSM, CCUG, NCPBB, ATCC, see Table 2.

Thermal stability of reassociated DNAs. The temperature at which 50% of reassociated DNA was hydrolysed by nuclease S1 (T_m) was determined by 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.

16S rRNA gene sequence determination. The almost complete 16S rRNA gene sequence was determined for strain CFML 96-195^T by direct PCR sequencing. DNA was amplified by using two 16S rRNA universal primers, pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGG-AGGTGATCCAGCCGCA-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 by using a model 480 DNA thermal cycler (Perkin-Elmer) by using a PCR

mixture (final vol. 100 µl) containing each of the four dNTPs at a concentration of 200 µM each, 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, pH (used for amplification), primer 59-195 (5'-CTTATTCTGTCGGTAACGTC-3'), primer 21-195 (5'-GGGCTCAACCTGGGAACGTC-3'), primer 15-195 (5'-TCCACCGCTTGTGCGGGCCCC-3') and primer 35-195 (5'-AGTTACCAGCCACGTCATGG-3'), covering the sequence at the following positions: 493-474, 608-627, 939-920 and 1116-1135, respectively (*E. coli* numbering; Brosius *et al.*, 1978).

Analysis of sequence data. The 16S rRNA gene sequence that we determined and the sequences of other pseudomonad reference strains obtained from the EMBL database (Table 3) were aligned and analysed by using the CLUSTAL X program (CLUSTAL X is a major rewrite of the multiple alignment program CLUSTAL W; Thompson *et al.*, 1994). 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 by using the bootstrap method (Felsenstein, 1985). A total of 1000 bootstrapped trees were generated. Calculations of levels of sequence similarity were based on the data for 1322 nt because deleted and unknown positions were eliminated.

Nucleotide sequence accession numbers. The accession numbers of the sequences used for comparison with the sequence that we determined are shown in Table 3.

RESULTS

Phenotypic and flagellation characteristics

Electron microscopic examination of strain CFML 96-195^T showed that the cells were motile by a single polar flagellum (Fig. 1). The width of the cells was 0.5 µm

Fig. 1. Electron micrograph of a cell of *P. libanensis* CIP 105460^T showing the single polar flagellum. Bar, 1 µm.

Table 4. Features differentiating *P. libanensis*, subclusters Vd and Ve, and the most important fluorescent species and biovars of the genus *Pseudomonas*

—, 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	<i>P. libanensis</i>	Subcluster Vd	Subcluster Ve	<i>P. aeruginosa</i>	<i>P. fluorescens</i> biovar I	<i>P. fluorescens</i> biovar II	<i>P. fluorescens</i> biovar III	<i>P. fluorescens</i> biovar IV	<i>P. fluorescens</i> biovar V	<i>P. chlororaphis</i>	<i>P. putida</i> biovar A	<i>P. putida</i> biovar B	<i>P. veronii</i>	<i>P. rhodesiae</i>	<i>P. mucidolens</i>	<i>P. synxantha</i>
Pyocyanin production	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
Denitrification	—	—	d	+	—	+	+	+	—	+	—	—	+	—	+	d
Nitrate reduction	+	—	—	+	+	+	—	d	d	+	+	+	+	+	+	+
Growth at 4 °C	+	+	+	—	+	+	+	+	d	+	d	+	+	+	+	d
Growth at 41 °C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lecithinase	+	d	—	+	+	d	+	+	d	d	—	—	—	—	d	d
Levan formation from sucrose	+	+	+	—	+	+	—	+	—	+	—	—	ND	+	+	—
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	+	+	+
2-Keto-D-glucamate	+	+	+	+	+	+	+	d	+	+	d	+	+	+	+	+
Mucate	+	+	+	—	+	+	d	+	+	+	d	+	d	+	+	+
Malonate	+	+	+	+	+	+	d	+	d	+	d	+	+	+	+	+
D-Tartrate	—	—	d	—	—	d	—	—	—	—	d	d	—	—	—	—
meso-Tartrate	d	—	d	—	—	d	d	—	d	—	d	—	—	—	—	—
Erythritol	+	—	d	—	d	d	+	—	d	—	—	—	+	—	+	—
D-Sorbitol	+	+	+	—	+	+	d	+	d	—	—	d	+	+	+	+
myo-Inositol	+	+	+	—	d	+	d	+	d	+	—	—	+	+	+	+
Adonitol	+	+	+	—	+	+	d	+	d	—	—	—	+	+	+	+
Benzozate	—	—	+	+	d	d	d	+	d	ND	d	+	+	—	—	—
Isoaconate	+	d	—	+	+	d	d	—	d	+	d	—	+	—	—	+
α-Aminobutyrate	+	+	+	—	+	—	—	—	—	—	—	—	+	—	—	+
L-Histidine	—	—	—	+	+	d	+	+	+	+	+	d	d	+	+	+
L-Tryptophan	—	—	—	d	+	d	d	—	d	+	—	+	+	—	+	—
Histamine	—	—	—	+	d	—	d	—	—	d	d	+	—	—	+	—
Tryptamine	—	—	d	—	—	d	d	—	—	—	d	+	—	—	—	—
Trigonelline	—	d	+	—	d	d	d	—	d	—	d	+	+	—	+	—

Table 5. Characteristics of the seven *P. libanensis* strains

+, Positive; –, negative. The number in parentheses indicates the number of strains that deviated from the most common result. All the reactions of the type strain are the same as the reactions of other *P. libanensis* strains except for the presence of trypsin.

Characteristic	Seven <i>P. libanensis</i> strains	Type strain
Conventional tests		
Urea	+	+
Carbon sources		
D-Trehalose	+	+
meso-Tartrate	–	–
N-Acetyl-D-glucosamine	+	+
α -L-Rhamnose	–	–
D-Lyxose	–	–
trans-Aconitate	+	+
Enzymic tests		
Lipase C ₁₄	–	–
Naphthol AS-BI phosphohydrolase	–	–
Valine arylamidase	+	+
Trypsin	+	–

and the length was 1 μ m. Phenotypic data (conventional, assimilation and enzymic tests) obtained for the seven strains of subcluster Vb are shown in Tables 4 and 5. Phenotypic tests, which proved useful for differentiating these strains from other *Pseudomonas* species and their biovars, are also listed in Table 4.

DNA relatedness and thermal stability

The results of DNA–DNA hybridizations obtained with labelled DNA of subcluster Vb strain CFML 96-195^T are shown in Tables 1 and 2. Hybridization values within subcluster Vb (8 strains) were 61–100%. For the three lowest relatedness values (61, 76 and 84%), the ΔT_m values were 8, 1 and 1 °C, respectively. Hybridization experiments were also performed between strain CFML 96-195^T and all 50 strains of the other phenotypic clusters and subclusters described previously as containing isolates from Lebanese spring waters (Dabboussi *et al.*, 1998); all values ranged from 18 to 67%. The highest values were obtained with subclusters Vd and Ve. The ΔT_m value varied between 6 and 9 °C (Table 1). The level of reassociation between strain CFML 96-195^T and all of the reference strains of the genus *Pseudomonas* used in this study (65 strains) are shown in Table 2. All values were less than 53% with ΔT_m values ranging from 6 to 8 °C.

DNA base composition

The G + C content of strains CFML 96-195^T, CFML 96-194 and CFML 96-183 was 58, 59 and 58 mol%, respectively (Table 1).

16S rDNA sequence analysis

The sequence of 1322 nt of the 16S rDNA gene of strain CFML 96-195^T was aligned to other sequences of all species of the genus *Pseudomonas sensu stricto* (Kerstens *et al.*, 1996) available from the database and with three newly described species: *P. veronii* (Elomari *et al.*, 1996), *P. rhodesiae* (Coroler *et al.*, 1996) and *P. monteilii* (Elomari *et al.*, 1997). A tree constructed by the neighbour-joining method (Saitou & Nei, 1987) showing phylogenetic relationships of strain CFML 96-195^T is shown in Fig. 2.

The levels of nucleotide similarities for the 16S rDNA sequence of strain CFML 96-195^T and other *Pseudomonas* species ranged from 94.3% (*P. aeruginosa*) to 99.5% (*Pseudomonas synxantha*). Furthermore, the sequence similarities between strain CFML 96-195^T and both strains CFML 96-170 (subcluster Vd) and CFML 96-198 (subcluster Ve) were 98.5 and 99.3%, respectively. Our 16S rRNA sequence comparison confirmed that CFML 96-195^T belongs to the genus *Pseudomonas*.

DISCUSSION

In this study, a total of eight *Pseudomonas* strains, grouped on the basis of a numerical analysis of phenotypic subcluster Vb (Dabboussi *et al.*, 1998), were subjected to further polyphasic characterization to determine their taxonomic status within the genus *Pseudomonas*.

The G + C content of strain CFML 96-195^T (58 mol%) falls within the expected range for *Pseudomonas* (58–70 mol%; Palleroni, 1984). The results of DNA–DNA hybridization experiments (when DNA of representative strain CFML 96-195^T of subcluster Vb was labelled) demonstrate that seven strains of this subcluster constitute a separate DNA hybridization group (76–100% hybridization). The current polyphasic species concept suggests only strains with approximately 70% or greater DNA–DNA relatedness and with a ΔT_m value of 5 °C or less constitute a single species (Wayne *et al.*, 1987). Only one strain of phenotypic subcluster Vb, strain CFML 96-210, had relative binding ratios of 61% with labelled DNA from strain CFML 96-195^T and a ΔT_m value of 8 °C. Therefore, this strain was expelled from the group. Furthermore, this strain was phenotypically distinguishable from other strains of subcluster Vb, since only this strain was able to denitrify, to assimilate 5-aminovaleate and was positive for tetra-thionate reductase. The high level of DNA–DNA relatedness between these seven strains is in agreement with the results of our phenotypic analysis which showed that they are closely related to each other. In contrast, strain CFML 96-195^T did not exhibit specific affinity to any other *Pseudomonas* species since the determined similarity values between the DNA of this strain and the DNA of other *Pseudomonas* strains were in the range 9–53% with ΔT_m values greater than 6 °C.

Fig. 2. Unrooted tree constructed by using the neighbour-joining method, showing the phylogenetic relationships of *P. libanensis* CIP 105460^T and other species of the genus *Pseudomonas sensu stricto*. The numbers indicate the percentage occurrence in bootstrapped trees.

The highest hybridization values were obtained with biovars I and V of *P. fluorescens* (51%) and with a representative strain of *Pseudomonas marginalis* (53%). Moreover, DNA–DNA hybridization levels between strain CFML 96-195^T and subcluster Vd and Ve strains were rather high (41–67%). In fact, strains of subcluster Vb (later named *P. libanensis*), Vd and Ve could be differentiated from each other by several phenotypic tests (Table 4). The derived 16S rRNA

sequence of strain CFML 96-195^T was aligned and compared with the sequences of strains CFML 96-170 and CFML 96-198 (subcluster Vd and Ve, respectively) and also with the sequences of other *Pseudomonas* species retrieved from the EMBL database. Strain CFML 96-195^T exhibited the lowest level of 16S rRNA sequence similarity with *P. aeruginosa* LMG 1242^T (94.3%). The highest level of sequence relatedness was observed with strain CFML 96-198

(subcluster Ve) and with *P. synxantha* IAM 12356^T (99.3 and 99.5%, respectively), but these organisms could be differentiated by DNA–DNA hybridization data (Table 2) and several phenotypic features (Table 4). In fact, rDNA sequences probably do not always contain enough information to ascertain taxonomy at the species level and additional studies of phenotypes and DNA–DNA hybridization are therefore necessary (Boivin-Jahns *et al.*, 1995; Wayne *et al.*, 1987). The phylogenetic tree shows that strain CIP 105460^T (= CFML 96-195^T) falls within the radiation of the genus *Pseudomonas* and specifically within the '*Pseudomonas fluorescens* intrageneric cluster' as defined by Moore *et al.* (1996). As a result of the polyphasic approach we propose a new species, *Pseudomonas libanensis* sp. nov., for the seven strains of subcluster Vb. Affiliation to the genus *Pseudomonas* is justified by the close phylogenetic relationships of strain CFML 96-195^T to other strains of this genus. The low level of DNA–DNA relatedness with other available *Pseudomonas* strains justifies the creation of a new species. *P. libanensis* strains are phenotypically and genotypically homogeneous and can be differentiated from related fluorescent members of the genus *Pseudomonas* by several phenotypic features (Table 4).

Differentiation of *P. libanensis* and *P. aeruginosa* is based on pyocyanin production, growth at 41 °C and assimilation of D-mannitol, mucate, sorbitol, benzoate and histamine.

P. fluorescens species (all biovars) are mainly differentiated from *P. libanensis* by the assimilation of α -aminobutyrate. *P. libanensis* and *Pseudomonas putida* biovar B differ phenotypically since all strains of *P. putida* biovar B produce lecithinase and assimilate histidine, adonitol, *myo*-inositol and erythritol, whereas strains of *P. libanensis* are unable to utilize these compounds. Differentiation of *P. libanensis* and *Pseudomonas mucidolens* is based on denitrification ability and assimilation of trigonelline, histamine, tryptophan and itaconate.

P. synxantha is differentiated from *P. libanensis* by levan formation and assimilation of histidine and erythritol. Finally, nitrate reduction, lecithinase production and the assimilation of erythritol, itaconate, trigonelline and benzoate were found to differentiate *P. libanensis* strains from Vd and Ve subcluster strains (Table 4).

Description of *Pseudomonas libanensis* sp. nov.

Pseudomonas libanensis (li.ba.nen'sis. L. n. *Libanus* a mountain area in Southern Syria, Lebanon; L. adj. *libanensis* from or of Lebanon).

Motile by a single polar flagellum as observed by transmission electron microscopy of a cell of the type strain. Gram-negative. Cells produce a fluorescent pigment on King B medium, but not phenazine pigments on King A medium. Growth occurs between 4 and 36 °C with optimal growth at 30 °C. Colonies on nutrient agar are smooth, circular and non-pigmented.

Poly- β -hydroxybutyrate is not accumulated as a carbon reserve material. Growth occurs in the presence of 3% NaCl but not in the presence of 7% NaCl. All strains reduce nitrate to nitrite and grow on cetrimide. Non-haemolytic on blood agar. Negative for lipase, elastase and tetrathionate reductase. Arginine dihydrolase, lecithinase, catalase and cytochrome oxidase are produced. Cells are able to form levan from sucrose and can use citrate and malonate. Indole and coagulase are not produced. Lysine and ornithine are not decarboxylated. L-Tyrosine is hydrolysed but not aesculin. 2,3,5-Triphenyltetrazolium chloride is not used. Negative Voges–Proskauer reaction and tributyrin test. No action against DNA or RNA. All strains utilize the following substrates as carbon and energy sources: D-arabitol, *myo*-inositol, L-arabitol, xylitol, D-mannitol, D-sorbitol, adonitol, erythritol, D-mannose, ribose, D-galactose, L-arabinose, D-xylose, D-glucosamine, ethanolamine, D-saccharate, mucate, 2-ketogluconate, protocatechuate, *p*-hydroxybenzoate, 3-hydroxybutyrate, malonate, D-glucuronate, D-galacturonate, itaconate and quinate. The following are not utilized as sole carbon and energy sources: maltitol, sucrose, α -L-fucose, D-turanose, α -D-melibiose, D-raffinose, maltotriose, maltose, D-cellobiose, β -gentiobiose, palatinose, D-tagatose, α -lactose, lactulose, 1-*O*-methyl- β -galactopyranoside, 1-*O*-methyl- α -galactopyranoside, 3-*O*-methyl-D-glycopyranose, 5-keto-D-gluconate, tricarballic acid, phenylacetate, gentisate, *m*-hydroxybenzoate, 5-aminovalerate, D-tartrate, benzoate, aesculin, trigonelline, histamine, tryptamine and inulin. All strains possess the following enzyme activities: alkaline phosphatase, acid phosphatase, esterase C₄ and leucine arylamidase. None of the strains possess the following enzyme activities: α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, α -mannosidase and α -fucosidase. Mean G + C content of the type strain is 58 mol%. All strains were isolated from Lebanese spring waters. No clinical significance is known. Type strain is CFML 96-195^T, deposited in the Collection de l'Institut Pasteur as CIP 105460^T.

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