Classification of *Ralstonia pickettii* biovar 3/'thomasi' strains (Pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica* sp. nov.

Thierry De Baere,¹ Sophia Steyaert,¹ Georges Wauters,² Paul De Vos,³ Johan Goris,³ Tom Coenye,³ Tetsushi Suyama,⁴ Gerda Verschraegen¹ and Mario Vaneechoutte¹

Author for correspondence: Mario Vaneechoutte. Tel: +32 9 240 36 92. Fax: +32 9 240 36 59.
e-mail: Mario.Vaneechoutte@rug.ac.be

¹ Department of Clinical Chemistry, Microbiology & Immunology, Blok A, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

² Medical Microbiology Unit, University of Louvain, Avenue Hippocrate 54, 1200 Brussels, Belgium

³ Laboratorium voor Microbiologie, Fac. Wetenschappen, Vakgroep WE10V, Ledeganckstraat 35, B-9000 Ghent, Belgium

⁴ Applied Microbiology Division, National Institute of Bioscience and Human Technology, 1-1 Higashi, Tsukuba-shi, Ibaraki 305-8566, Japan

Strains isolated independently from two patients could be recognized as *Ralstonia pickettii* biovar 3/'thomasi'. The 16S rDNA sequences of these strains and two other strains of *R. pickettii* biovar 3/'thomasi' clustered at less than 98% similarity versus all other described *Ralstonia* species and at less than 97% versus the two other *R. pickettii* biovars. The separate species status of *R. pickettii* biovar 3/'thomasi' was confirmed by DNA–DNA hybridization, indicating less than 60% DNA homology with the *R. pickettii* biovars Va-1 and Va-2 and with two as-yet unclassified but biochemically similar *Ralstonia* strains. Phenotypically, this *Ralstonia* species can be distinguished from all described *Ralstonia* species by its acidification of D-arabitol and mannitol and by its lack of nitrate reduction and of alkalization of tartrate and from two as-yet unclassified *Ralstonia* strains only by its lack of nitrate reduction. The name *Ralstonia mannitolytica* sp. nov. is proposed, reflecting the characteristic acidification of mannitol. Resistance to desferrioxamine is another difference from *R. pickettii* and *Ralstonia solanacearum*. Although several nosocomial outbreaks have been associated with *R. mannitolytica*, life-threatening infections have not yet been reported, possibly due to misidentification as *Pseudomonas fluorescens* or *Burkholderia cepacia*. In at least one of the two cases reported here, the *R. mannitolytica* isolate was found to be clinically relevant, causing recurrent nosocomial meningitis, with an infected implanted catheter as the source. The type strain of *R. mannitolytica* is NCIMB 10805^T (= LMG 6866^T), which was isolated during the first described outbreak as '*Pseudomonas thomasi*' at St Thomas' Hospital, London, UK, in 1971.

Keywords: *Ralstonia pickettii* biovar 3/'thomasi', *Ralstonia mannitolytica* sp. nov., infection

INTRODUCTION

The genus *Ralstonia* (Yabuuchi *et al.*, 1995) has been created for a group of organisms from ecologically diverse niches to accommodate bacteria that were formerly classified as members of *Burkholderia*

(Yabuuchi *et al.*, 1992) and *Alcaligenes*. The type species of the genus, *Ralstonia pickettii*, was originally regarded as the only representative of clinical importance (Dimech *et al.*, 1993; Fass & Barnishan, 1976; Fujita *et al.*, 1981; Gardner & Shulman, 1984; Kahan *et al.*, 1983; Lacey & Want, 1991; Raveh *et al.*, 1993; Roberts *et al.*, 1990; Verschraegen *et al.*, 1985). Only recently, the taxonomy of this genus has begun to be elucidated with the description of several new species, *Ralstonia paucula*, *Ralstonia gilardii* and *Ralstonia basileensis*, some of which are of moderate

Abbreviation: TSA, tryptic soy agar.

The GenBank/EMBL/DBJ accession numbers for the 16S rDNA sequences reported in this paper are AJ270252–AJ270266 and AJ271437.

clinical importance (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Moissenet *et al.*, 1999; Osterhout *et al.*, 1998; Steinle *et al.*, 1998).

Historically, *R. pickettii* was created for a group of clinical isolates (Ralston *et al.*, 1973) and also turned out to include strains of CDC group Va-2 (Tatum *et al.*, 1974; Riley & Weaver, 1975) and possibly other strains. The CDC groups Va-2 and Va-1 were regarded as two different biovars and hence Pickett & Greenwood (1980) decided that CDC groups Va-1 and Va-2 represented two different biovars of *R. ('Pseudomonas') pickettii*. Shortly before this, King *et al.* (1979) had concluded that *R. ('Pseudomonas') pickettii* contained several biovars, including the strains originally isolated from clinical samples in St Thomas' Hospital, London, UK (Phillips & Eykyn, 1972; Phillips *et al.*, 1972), and which had been designated '*Pseudomonas thomasi*'. Although this name was never validated, it has been used several times (Baird *et al.*, 1976; King *et al.*, 1979; Costas *et al.*, 1990). According to Pickett (1994), the present taxonomic situation of *R. pickettii* should be reduced to the recognition of three biovars, with biovar 1 equal to CDC group Va-1, biovar 2 equal to CDC group Va-2 and biovar 3 mostly consistent with the invalid '*Pseudomonas thomasi*' (Pickett, 1994).

Here, we report a case of recurrent nosocomial meningitis caused by a colistin-resistant, Gram-negative rod that was identified by means of API 20NE as *Pseudomonas fluorescens*. Because of its colistin resistance, this strain was studied in more detail, which lead to its identification as *R. pickettii* biovar 3/'*thomasi*'. The further data gathered, by means of sequence determination of the 16S rDNA, determination of the cellular fatty acid composition, DNA hybridization, SDS-PAGE and extensive characterization of biochemical reactivity, indicate that this biovar can be considered as a separate species within the genus *Ralstonia*, named *Ralstonia mannitolytica* sp. nov. after its characteristic acidification of mannitol, distinct from all other described *Ralstonia* species. Retrospectively, a strain stored previously as *P. fluorescens* and isolated repeatedly from an abdominal haematoma of a patient with cholangiocarcinoma and a strain from an outbreak due to contaminated water in the neonatal intensive care department of the Ghent University Hospital, Belgium (GUH), in 1990 could also be identified as *R. mannitolytica*. Several nosocomial outbreaks with *R. pickettii* biovar 3/'*thomasi*', mostly due to contaminated fluids, have been described (Costas *et al.*, 1990; Pan *et al.*, 1992; Phillips *et al.*, 1972), but no life-threatening infections have been reported yet to our knowledge.

METHODS

Bacterial strains. The strains studied are listed in Table 1. The clinical strains LMG 19090, LMG 19091 and LMG 19092 were isolated at the GUH. Strain LMG 19090 (LBV407/UCL310) was isolated repeatedly from a patient with

recurrent meningitis (patient 1), strain LMG 19091 (LBV371/RAL13) was isolated repeatedly from an abdominal haematoma of a patient with cholangiocarcinoma (patient 2) and strain LMG 19092 (PSE061) was one of several strains isolated during an outbreak due to contaminated water at the GUH neonatal intensive care department in 1990. Strains LMG 19083 (RAL05/ML7), LMG 19087 (RAL07/YL13), LMG 19088 (RAL06/ML10) and LMG 19089 (RAL04/MC5) are environmental soil isolates described by Suyama *et al.* (1998) and were included because some of these strains showed the highest 16S rDNA sequence similarity to the clinical strains of all the sequences available from the GenBank/EMBL database. All other strains studied here, including the *R. mannitolytica* type strain (LMG 6866^T), were supplied by the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were isolated at the GUH. [*Pseudomonas*] *syzygii* strains, which are so closely related to *Ralstonia solanacearum* (Brim *et al.*, 1998; this study) that they can be regarded as members of this genus, were included to exclude synonymy with the *R. pickettii* biovar 3/'*thomasi*' strains studied here.

Biochemical characterization. Growth at 30 and 37 °C was evaluated on tryptic soy agar (TSA) (Sanofi Diagnostics Pasteur). Growth at 42 °C was tested by adding two drops of a McFarland suspension 3 in water, prepared from a 24 h culture on TSA, to 5 ml of tryptic soy broth and subsequent incubation in a water bath at 42 °C and reading after 48 h. Conventional tests were carried out as described elsewhere (Gilligan, 1995). Commercial tests (API ZYM, API 20NE, API 50CH and ID 32GN; bioMérieux) were carried out according to the instructions of the manufacturer. Assimilation testing was carried out using the AUX medium (bioMérieux). To test for assimilation and alkalization of organic substrates, Simmons' citrate agar was used, whereby citrate was replaced by organic substrates at a concentration of 0.2% (w/v). Desferrioxamine susceptibility was tested according to a method adapted from Lindsay & Riley (1991), by loading 6 mm diameter paper discs with 250 µg desferal on Müller Hinton II agar (MHA) (BBL Becton Dickinson). Alkaline phosphatase, pyrrolidonyl arylamidase and benzyl arginine arylamidase (trypsin) were tested using Rosco tablets (Taastrup, Denmark) and read after 4 h. Colistin susceptibility was tested using 10 µg colistin paper disks (BBL) on MHA and susceptibility to the vibriostatic agent O:129 was tested with Sanofi Diagnostics Pasteur paper discs on MHA. Acidification of ethylene glycol was tested as described previously (Wauters *et al.*, 1998). Staining of flagella was done as described previously (Kodaka *et al.*, 1982).

16S rDNA sequence determination. Amplification was done by PCR with primers named $\alpha\beta$ NOT (5'-AGTTTGATC-CTGGCTCAG-3') and ω MB (5'-TACCTTGTTACGAC-TTCGTCCCA-3'). The reactions were performed in a final reaction mixture of 50 µl containing 25 µl Master Mix (Qiagen), 0.2 µM of each primer and 5 µl of a DNA suspension obtained by alkaline lysis. The amplification reactions were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems) with the following cycling parameters: 94 °C for 5 min, followed by three cycles of 45 s at 94 °C, 2 min at 50 °C and 1 min at 72 °C and 30 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. The amplification products were checked by 2% agarose gel electrophoresis and staining with ethidium bromide. The amplification products were purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions. Sequencing

Table 1 List of strains studied

Names of taxa in brackets indicate that the organisms were misnamed at the corresponding taxonomic level. Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CDC, Centers for Disease Control, Atlanta, GA, USA; CIP, Collection Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; GUH, Ghent University Hospital, Belgium; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, UK; NCPPB, National Collection of Plant-pathogenic bacteria, Harpenden Laboratory, Herts, UK; UCL, Université Catholique de Louvain, Belgium.

Strain	Other strain designation(s)	Origin
[<i>P.</i>] <i>syzygii</i>		
LMG 6969	RAL08, Eden-Green S593	
LMG 6970	RAL09, Eden-Green S594	
LMG 10661 ^T	RAL10 ^T , Eden-Green R001 ^T , Jones W1330 ^T , NCPPB 3446 ^T	West Sumatra (' <i>Syzygium aromaticum</i> ') West Java
LMG 10662	RAL11, Eden-Green R002, NCPPB 3445	
<i>R. basilensis</i>		
LMG 18990*	DSM 11853, Steinle RK1	Laboratory fixed bed reactor, 2,6-dichlorophenole as sole energy source
<i>R. eutropha</i>		
LMG 1194	RAL14, CCUG13796, DSM 515, Schlegel B19	USA, forest soil
LMG 1199 ^T	RAL15 ^T , ATCC 17697 ^T , CCUG 1776 ^T , DSM 531 ^T , ICPB 3984 ^T , NCIB 11842 ^T	USA, soil
LMG 1201	RAL16, ATCC 17699, ATCC 23440, CCUG 13797, DSM 428, NCIB 10442, Schlegel H16	Göttingen, Germany, source sludge
<i>R. gilardii</i>		
LMG 5886 ^T *	API 141-2-84 ^T , ATCC 700815 ^T , Gilardi 4325 ^T	Whirlpool
<i>R. mannitolytica</i>		
LMG 6866 ^T	NCIB 10805 ^T	St. Thomas' Hospital, London, UK, 1971, blood
LMG 19090	LBV407, UCL310	GUH, 1998, recurrent meningitis
LMG 19091	RAL13, LBV371, GUH 98 06 3523	GUH, 1998, abdominal haematoma
LMG 19092	PSE061, GUH S90 03985	GUH, 1990, sputum
<i>R. paucula</i>		
LMG 3244 ^T	ATCC 700817 ^T , CCUG 12507 ^T , CDC E6793 ^T	Oregon, USA, human respiratory tract
LMG 3515	CCUG 12411, CCUG 12505, CDC E8967, LMG 3245	Georgia, USA, sputum
<i>R. pickettii</i>		
LMG 7001*	CCUG 3314, Pickett K-214	USA, Sephadex eluate
LMG 7002*	CCUG 3316, LMG 7146, Pickett K-279	
LMG 7008*	CCUG 2165	Göteborg, Sweden, 1973, urine
LMG 7145*	CCUG 3315, Pickett K-232	
<i>R. pickettii</i> biovar Va-1		
LMG 7005	CCUG 1467, CDC A5832, PSE056	
LMG 7014	RAL02, CCUG 12491, Gilardi GLG 3963	Blood
LMG 7015	RAL03, CCUG 12492, Gilardi GLG 3905	Blood
LMG 7160	RAL01, CIP 74.22, CIP R866	Blood culture
LMG 19083	ML7, RAL05	Soil, Japan
LMG 19084	PSE058, GUH S89 04031	GUH, 1989, sputum
LMG 19086	PSE060, GUH S89 04266	GUH, 1989, sputum
LMG 19088	ML10, RAL06	Soil, Japan
<i>R. pickettii</i> biovar Va-2		
ATCC 27512	WG076, Pickett K-550	Patient with tracheotomy, 1973
LMG 5942 ^T	ATCC 27511 ^T , CCUG 3318 ^T , CIP 73.23 ^T , ICPB 3981 ^T , JCM 5969 ^T , NCTC 11149 ^T , Pickett K-288 ^T , PSE055 ^T , WG071 ^T	Los Angeles, CA, USA, patient with tracheotomy
LMG 19085	RAL12, GUH 99 03 4045	GUH, 1999, nebulizer
<i>R. solanacearum</i>		
LMG 2299 ^T	RAL17 ^T , CCUG 14272 ^T , DSM 9544 ^T , JCM 10489 ^T , Kelman 60-1 ^T , NCPPB 325 ^T	USA, <i>Lycopersicon esculentum</i>
LMG 2303	RAL18, NCPPB 078	Honduras
<i>Ralstonia</i> sp.		
LMG 19087	YL13, RAL07	Japan, soil
LMG 19089	MC5, RAL04	Japan, soil

* Strain was only included in SDS-PAGE of whole-cell proteins.

was performed on an ABI 310 sequencer using the ABI Big Dye cycle sequencing reaction kit with AmpliTaq FS DNA polymerase (Perkin Elmer) with primers described previously (Coenye *et al.*, 1999). Analysis of the sequences and clustering was done by using GENE COMPAR version 4.1 (Applied Maths).

DNA base composition. DNA was degraded enzymatically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermo-stabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH

4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma-Aldrich) was used as the calibration reference.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements (excitation filter of 360 nm, emission filter of 465 nm). The optimal renaturation temperature was determined according to the equation of De Ley *et al.* (1970).

PAGE of cell proteins. Cells were grown for 48 h on TSA at 37 °C. SDS protein extracts were prepared and electro-

Table 2 Phenotypic characteristics useful in the differentiation of the *Ralstonia* species

The following taxa were included in the analysis: 1, *R. mannitolytica* strains LMG 6866^T, LMG 19090, LMG 19091 and LMG 19092; 2, *R. pickettii* biovar Va-1 strains LMG 7014, LMG 7015, LMG 7160, LMG 19083, LMG 19084, LMG 19086 and LMG 19088; 3, *R. pickettii* biovar Va-2 strains ATCC 27512, LMG 5942^T and LMG 19085; 4, *Ralstonia* sp. strain LMG 19089; 5, *Ralstonia* sp. strain LMG 19087; 6, *R. solanacearum* strains LMG 2299^T and LMG 2303; 7, *R. eutropha* strains LMG 1194, LMG 1199^T and LMG 1201; 8, *R. paucula* strains LMG 3515 and LMG 3244^T; and 9, *R. gilardii*. Data for *R. gilardii* were taken from Coenye *et al.* (1999). R, Resistant; S, susceptible; NK, not known. Growth is scored as: v, variable; +, all strains positive; (+), weakly positive; —, all strains negative.

Characteristic	1	2	3	4	5	6	7	8	9
Flagellation	Polar 1	Polar 1	Polar 1	Polar 1	Polar 1	None	Peritrichal	Peritrichal	Polar 1
Reaction to:									
Colistin	R	R	R	R	R	R	S	S	NK
Desferrioxamine	R	S	S	R	R	S	R	R	NK
Viability on TSA at room temperature	< 6 d	< 6 d	< 6 d	< 6 d	< 6 d	< 6 d	> 21 d	> 21 d	NK
Growth at 42 °C	+	V	V	+	—	—	V	V	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	—
Alkaline phosphatase (Rosco)	—	—	—	—	—	—	+	—†	++‡
Pyrrrolidonyl arylamidase	+	+	+	+	+	—	+	+	NK
Phenylalanine deaminase	v§	v§	+	v§	—	—	V	—	NK
Urease	+	+	+	+	+	+	—	+	—
Nitrate reduction	—	+	+	+	+	+	V	—	—
Tween 80 hydrolysis	+	+	+	+	+	+	V	+	NK
Production of acid from:									
L-Arabinose	+	+	+	+	+	—	—	—	—
D-Arabitol	+	—	—	+	+	—	—	—	NK
Glucose	+	+	+	+	+	+	—	—	NK
Inositol	—	—	—	—	—	+	—	—	NK
Lactose	+	+	—	+	+	—	—	—	NK
Maltose	+	+	—	+	+	—	—	—	NK
Mannitol	+	—	—	+	+	—	—	—	NK
Sucrose	—	—	—	—	—	+	—	—	NK
D-Xylose	+	+	+	+	+	—	—	—	—
Alkalinization of:									
Acetate	+	+	+	+	+	—	+	+	NK
Allantoin	—	—	—	—	—	—	+	—	NK
Citrate	+	+	+	+	+	+	+	+	—
Galacturonate	+	+	+	+	+	+	—	—	NK
Lactate	+	+	+	+	+	—	+	+	NK
Maleate	—	+	+	—	(+)	V	V	V	NK
Malonate	+	+	+	+	+	—	+	+	NK
Mucate	+	+	+	+	+	+	—	+	NK
Oxalate	—	—	—	—	—	—	+	+	NK
Tartrate	—	+	+	—	—	+	+	+	NK
Assimilation of:									
3-Hydroxybenzoate	—	—	—	—	—	—	+	—	NK
Caprate	+	+	+	+	+	—	+	+	—
Propionate	+	+	+	+	+	—	+	+	NK
Suberate	+	+	+	+	+	—	+	+	NK

* Negative according to Vandamme *et al.* (1999).

† Negative with Rosco tablets, containing nitrophenyl phosphate as the substrate. Vandamme *et al.* (1999) reported a positive reaction, using API ZYM (naphthyl phosphate as the substrate). In this study, all strains tested with API ZYM were positive.

‡ Tested with API ZYM.

§ Phenylalanine deaminase reactions were difficult to interpret (weakly positive) and very variable.

phoresed according to Pot *et al.* (1994) and the collected data were interpreted as described by Vauterin & Vauterin (1992). The similarity between all pairs of electrophoresis patterns was calculated by the Pearson product-moment correlation coefficient, expressed as a percentage.

Determination of cellular fatty acid composition. Cellular fatty acids were extracted from cells grown for 48 h on TSA at 37 °C. The fatty acid analysis was performed at 45–210 °C by using a capillary column CP-Sil 88 (Chrompack International) and a Delsi DI 200 chromatograph (Intersmat) equipped with split-splitless injection and a flame-ionization detector. The results were expressed as relative percentages

by using an Enica 31 integrator obtained from Delsi Nermag Instruments (Intersmat).

RESULTS

Microbiology

Strain LMG 19090, obtained from patient 1, was isolated on *Burkholderia cepacia* selective medium (Mast Diagnostics), containing 100 mg ticarcillin l⁻¹ and 300 U polymyxin B ml⁻¹. Testing with API 20NE resulted initially in identification as *P. fluorescens*

Table 3 Cellular fatty acid composition (mol %)

Taxon	Strains	16:1 ^{9cis}	16:0	18:1 ^{11cis}	14:0
<i>R. mannitolytica</i>	LMG 6866 ^T , LMG 19090, LMG 19091	30–38	21–30	7–15	5–7
<i>R. pickettii</i> biovar Va-1	LMG 7160, LMG 7014, LMG 19084	41–42	26–28	13–15	10
<i>R. solanacearum</i>	LMG 2299 ^T , LMG 2303	41–42	26–33	9–13	6–10
<i>R. eutropha</i>	LMG 1194, LMG 1199 ^T , LMG 1201	41–46	27–30	14–18	4–6
<i>R. paucula</i>	LMG 3515, LMG 3244 ^T	42–44	22–23	17–20	6–7

Table 4 DNA G + C content and DNA–DNA binding values of strains examined

Taxon	G + C content (mol%)	DNA–DNA binding with DNA from (%):								
		1	2	3	4	5	6	7	8	9
1. <i>R. mannitolytica</i> LMG 6866 ^T	66.2	100								
2. <i>R. mannitolytica</i> LMG 19090	66.2	88	100							
3. <i>R. mannitolytica</i> LMG 19091	66.2	86	100	100						
4. <i>R. pickettii</i> biovar Va-2 LMG 5942 ^T	64.0	58	ND	ND	100					
5. <i>R. pickettii</i> biovar Va-2 LMG 19085	64.1	53	48	52	85	100				
6. <i>Ralstonia</i> sp. LMG 19089	65.1	56	58	57	64	64	100			
7. <i>R. pickettii</i> biovar Va-1 LMG 19083	64.1	46	ND	ND	57	62	66	100		
8. <i>R. pickettii</i> biovar Va-1 LMG 19088	64.1	50	ND	ND	61	61	68	100	100	
9. <i>Ralstonia</i> sp. LMG 19087	64.0	50	ND	ND	63	63	71	65	69	100

ND, Not done.

(profile code 0 054 555). Strain LMG 19091, obtained from patient 2, had been identified previously as *P. fluorescens* (API profile code 0 045 555). Strain LMG 19092 (PSE061) was isolated in 1990 from the sputum of a neonate during an outbreak in the paediatric department and had been identified at that time as *R. pickettii*. The combined features of biochemical characteristics and colistin resistance indicated that these isolates were strains of *R. mannitolytica* sp. nov. (formerly *Ralstonia pickettii* biovar 3/‘*thomasii*’) (Clark *et al.*, 1984; Gilligan, 1995).

The biochemical characteristics useful for the differentiation of *Ralstonia* species are summarized in Table 2. The three clinical *R. mannitolytica* strains were motile by a single polar flagellum, while motility was not observed for the culture collection *R. mannitolytica* type strain LMG 6866^T. During this study, it was observed that freshly isolated strains were very motile and that motility decreased upon prolonged preservation and subculture. The type strain of *R. mannitolytica*, LMG 6866^T, was non-motile, possibly as a consequence of prolonged preservation.

All four strains grew at 30, 37 and 42 °C and were viable for less than 6 d on TSA at room temperature. Oxidase and catalase were positive. They were resistant to desferrioxamine, O:129 and colistin. No acid was produced from ethylene glycol. Urease, pyrrolidonyl arylamidase (Rosco), Tween esterase and phenylalanine deaminase were positive. Nitrate reduction,

indole and hydrogen sulfide production, alkaline phosphatase (Rosco), arginine dihydrolase, lysine and ornithine decarboxylases and aesculin and gelatin hydrolysis were negative. Acid was produced oxidatively from glucose, L-arabinose, lactose, maltose, mannitol, D-arabitol and D-xylose. Alkalinization occurred on minimal mineral agar with acetate, serine, malonate, β-alanine, 4-aminobutyrate, azelate, succinate, fumarate, butyrate, formate, malate, mucate, galacturonate, citrate, histidine and lactate, but not with acetamide, adipate, alginate, allantoin, amygdalin, L-arginine, benzoate, L-ornithine, maleate or tartrate.

Testing by means of API 20NE, API 50CH and ID 32GN indicated that the strains assimilated acetate, N-acetylglucosamine, L-alanine, L-arabinose, D-arabitol, 4-hydroxybenzoate, 3-hydroxybutyrate, caprate, citrate, fructose, galactose, gluconate, glucose, 2-keto-gluconate, glycerol, histidine, D-lactate, malate, malonate, mannitol, L-proline, propionate, serine, suberate and D-xylose, but not adipate, amygdalin, L-fucose, glycogen, inositol, itaconate, 5-ketogluconate, maltose, mannose, melibiose, phenylacetate, rhamnose, ribose, sucrose, salicin, D-sorbitol or 3-hydroxybenzoate and confirmed the absence of nitrate reduction.

Using the API ZYM system, the following enzymes were detected: C4 esterase, C8 esterase-lipase, C14 lipase, leucine arylamidase, acidic and alkaline phos-

Table 5 16S rDNA sequences included in this study

Taxon	16S rDNA accession no.	No. of nucleotides sequenced	Reference(s)
[<i>P.</i>] <i>syzygii</i>	AB021403	1513	–
[<i>P.</i>] <i>syzygii</i> LMG 10661 ^T	AJ270252	469	This study
[<i>P.</i>] <i>syzygii</i> LMG 6970	AJ270253	464	This study
[<i>P.</i>] <i>syzygii</i> LMG 10662	AJ270254	463	This study
[<i>P.</i>] <i>syzygii</i> LMG 6969	AJ270255	449	This study
<i>R. eutropha</i>	M32021	1511	–
<i>R. gilardii</i>	AF076645	1451	Coenye <i>et al.</i> (1999)
<i>R. mannitolytica</i> LMG 19091	AJ270256	1453	This study
<i>R. mannitolytica</i> LMG 19090	AJ270257	1450	This study
<i>R. mannitolytica</i> LMG 19092	AJ271437	517	This study
<i>R. mannitolytica</i> LMG 6866 ^T	AJ270258	1449	This study
<i>R. paucula</i>	AF085226	1484	Vandamme <i>et al.</i> (1999)
<i>R. pickettii</i> bv. Va-1 LMG 7160	AJ270260	1450	This study
<i>R. pickettii</i> bv. Va-1 LMG 19083	AB013428	441	This study, Suyama <i>et al.</i> (1998)
<i>R. pickettii</i> bv. Va-1 LMG 19088	AB013429	447	This study, Suyama <i>et al.</i> (1998)
<i>R. pickettii</i> bv. Va-1 LMG 19084	AJ270261	342	This study
<i>R. pickettii</i> bv. Va-1 LMG 7015	AJ249848	458	This study
<i>R. pickettii</i> bv. Va-1 LMG 7005	AJ270262	424	This study
<i>R. pickettii</i> bv. Va-1 LMG 7014	AJ270264	472	This study
<i>R. pickettii</i> bv. Va-1 LMG 19086	AJ270263	439	This study
<i>R. pickettii</i> bv. Va-1	X70348	294	Seal <i>et al.</i> (1993)
<i>R. pickettii</i>	AB004790	1468	–
<i>R. pickettii</i> bv. Va-2 LMG 5942 ^T	AJ270265	474	This study
<i>R. pickettii</i> bv. Va-2 LMG 5942 ^T	S55004	970	Yabuuchi <i>et al.</i> (1992)
<i>R. pickettii</i> bv. Va-2 ATCC 27512	X67042	1417	Li <i>et al.</i> (1993)
<i>R. pickettii</i> bv. Va-2 LMG 19085	AJ270266	450	This study
<i>R. pickettii</i> bv. Va-2	X70349	294	Seal <i>et al.</i> (1993)
<i>R. solanacearum</i>	X67036	1434	Li <i>et al.</i> (1993)
<i>Ralstonia</i> sp. LMG 19089	AJ270259	1455	This study, Suyama <i>et al.</i> (1998)
<i>Ralstonia</i> sp. LMG 19087	AB01438	447	This study, Suyama <i>et al.</i> (1998)
CDC group IVC-2 strain	AF098288	1495	Moissenet <i>et al.</i> (1999)
CDC group IVC-2 strain	AF067657	1527	Osterhout <i>et al.</i> (1998)
<i>Alcaligenes faecalis</i>	M22508	1475	Dewhirst <i>et al.</i> (1989)
<i>Brevundimonas diminuta</i>	M59064	1445	–
<i>Burkholderia andropogonis</i>	X67037	1435	Li <i>et al.</i> (1993)
<i>Burkholderia caryophylli</i>	X67039	1420	Li <i>et al.</i> (1993)
<i>Burkholderia cepacia</i>	M22518	1473	Dewhirst <i>et al.</i> (1989)
<i>Burkholderia gladioli</i>	X67038	1434	Li <i>et al.</i> (1993)
<i>Burkholderia mallei</i>	S55008	378	Yabuuchi <i>et al.</i> (1992)
<i>Burkholderia mallei</i>	S55000	962	Yabuuchi <i>et al.</i> (1992)
<i>Comamonas testosteroni</i>	M11224	1563	Yang <i>et al.</i> (1985)
<i>Pseudomonas aeruginosa</i>	X06684	1537	Toschka <i>et al.</i> (1988)

bv., Biovar.

phatase and phosphoamidase. Valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase were not detected.

Cellular fatty acid composition

The cellular fatty acid composition was determined for *R. mannitolytica* strains LMG 6866^T, LMG 19090 and LMG 19091. Table 3 presents the data in comparison

with those obtained for other *Ralstonia* species. The principal components for the *R. mannitolytica* strains were 16:1^{9cis} (range 30–36 mol%) and 16:0 (range 21–30 mol%), with 7–15 mol% of 18:1^{11cis} and 5–7 mol% of 14:0.

DNA G + C content

The DNA G + C content for all three *R. mannitolytica* strains tested was 66.2 mol% (Table 4), which was higher than the values obtained for the four *R. pickettii*

Fig. 1. Unweighted pair group method with averages (UPGMA) cluster analysis of the 16S rDNA sequences. Accession numbers indicated with asterisks refer to sequences determined in this study. The allocation of strains to either *R. pickettii* biovar Va-1 or Va-2 was done by testing acid production from maltose and lactose using OF medium: both positive for *R. pickettii* biovar Va-1 and both negative for *R. pickettii* biovar Va-2 (Pickett, 1994).

strains (64.0–64.1%) and for the two unnamed *Ralstonia* strains (64.0 and 65.1%).

16S rDNA sequence determination and total DNA homology

The 16S rDNA sequences included in the analysis are listed in Table 5. The 16S rDNA sequences of the three clinical strains LMG 19090, LMG 19091 and LMG 19092 were identical and clustered at more than 99.5% sequence similarity with the *R. mannitolytica* type strain, LMG 6866^T (Fig. 1). DNA–DNA hybridization confirmed that these three strains belonged to the same species (Table 4).

Closest to this group, with a 16S rDNA sequence similarity of more than 99%, was strain LMG 19089 (MC5), which was originally isolated from a brown lowland soil (pH 6.0) near the sandy bank of the Kinu river, Hama, Mitsuakido-Shi, Ibaraki Prefecture, Japan, and which was found to degrade poly(hexamethylene carbonate) and poly(ϵ -caprolactone) and to accumulate poly(3-hydroxybutyrate) granules inside the cells (Suyama *et al.*, 1998). However, the level of DNA–DNA hybridization with three *R. mannitolytica* strains was only 56–58% (Table 4), so it can be concluded that this strain probably belongs to a separate, as-yet unclassified species.

The *R. solanacearum* 16S rDNA sequence (GenBank

Fig. 2. Digitized representation of normalized SDS-PAGE protein profiles of different *Ralstonia* species and a dendrogram derived from the UPGMA linkage of correlation coefficients between the SDS-PAGE protein profiles of the strains studied. The zones indicated by arrowheads corresponding to molecular masses of approximately 60 and 80 kDa were omitted from the clustering.

accession no. X67036) clustered at more than 99% similarity with the sequences obtained for the [*P.*] *syzygii* strains LMG 6969, LMG 6970, LMG 10661^T and LMG 10662 (Fig. 1). LMG 19089 and the *R. mannitolytica* strains clustered with *R. solanacearum*/[*P.*] *syzygii* at less than 98% rDNA sequence similarity. The 16S rDNA sequences for the *R. pickettii* biovar Va-1 and Va-2 strains clustered at 96% similarity versus the LMG 19089–*R. mannitolytica*–*R. solanacearum*/[*P.*] *syzygii* cluster. The sequences of *R. paucula*, *R. gilardii* and *Ralstonia eutropha* strains apparently formed a separate cluster at a similarity of less than 96% to *R. pickettii*, *R. solanacearum*/[*P.*] *syzygii*, strain LMG 19089 and *R. mannitolytica*. The available 16S rDNA sequences of *Ralstonia* group CDC IVc-2 strains [GenBank accession nos AF098288 (Moissenet *et al.*, 1999) and AF067657 (Osterhout *et al.*, 1998)] were found to be 100% identical to that of a *R. paucula* strain (Vandamme *et al.*, 1999; GenBank accession no. AF085226). Sequence determination of the 5' end of the 16S rDNA revealed that the three *R. pickettii* strains found to produce no acid from maltose and lactose (i.e. *R. pickettii* biovar Va-2 strains) differed from the eight maltose and lactose acidification-positive *R. pickettii* strains (i.e. *R. pickettii* biovar Va-1 strains) at two positions. At *Escherichia coli* position 259, the A observed for *R. pickettii* biovar Va-2 is replaced by G for *R. pickettii* biovar Va-1. The other transition is at *E. coli* position 269, at which the T in the 16S rDNA sequence of *R. pickettii* biovar Va-2 is replaced by an A in *R. pickettii* biovar Va-1. The differences in biochemistry and 16S rDNA sequence are corroborated by the DNA–DNA hybridization data, since the two Japanese soil isolates (LMG 19083

and LMG 19088), which were identified as *R. pickettii* biovar Va-1 according to 16S rDNA sequence and biochemical characteristics, showed only 57–62% DNA relatedness to the two *R. pickettii* biovar Va-2 strains included in the DNA–DNA hybridization studies (Table 4).

PAGE of cell proteins

SDS-PAGE largely confirmed the 16S rDNA sequence and the DNA–DNA hybridization data. Strains LMG 6866^T, LMG 19090 and LMG 19091 belonged to a cluster with internal similarity of more than 80%, well separated from all other reference and non-reference strains. This grouping was only obtained when two small zones at 60 kDa (± 1 kDa) and 80 kDa (± 1 kDa), which contained a heavy protein band in the patterns of strains LMG 19090 and LMG 19091, were excluded from the similarity calculations (Fig. 2).

DISCUSSION

Clinical relevance of *R. mannitolytica*

A limited number of cases of hospital outbreaks with '*Pseudomonas thomasi*' and *R. pickettii* biovar 3/'*thomasi*' isolates have been reported in the literature (Baird *et al.*, 1976; Phillips *et al.*, 1972; Dowsett, 1972; Pan *et al.*, 1992). The first report (Phillips *et al.*, 1972) dealt with bacteraemia and bacteriuria in 25 patients due to the administration of parenteral fluids prepared at the hospital pharmacy, where deionized water contaminated with '*P. thomasi*' had been used (Phillips *et al.*, 1972; Phillips & Eykyn, 1972). Pan *et al.* (1992) reported that 23 of 39 *R. pickettii* isolates from

an epidemic involving 24 patients that was caused by contaminated saline solution (prepared by the hospital pharmacy) belonged to '*P. thomasii*'.

The clinical importance of *R. mannitolytica* may have been overlooked, possibly due to misidentification as *P. fluorescens*, which does not lead to further investigation since this organism is usually considered to be a contaminant. Indeed, after strain LMG 19090, initially identified as *P. fluorescens* (API profile code 0 054 555), had been recognized as *R. mannitolytica* because of colistin resistance and further biochemical testing, we studied retrospectively the only other strain (LMG 19091) in the GUH collection that had been stored as *P. fluorescens* (API profile code 0 045 555) and could identify it also as *R. mannitolytica*. Thus, microbiologists should consider the possibility of an identification as *R. mannitolytica* for colistin-resistant *P. fluorescens* isolates. Strain LMG 19092 belonged to a collection of strains that had been catalogued previously as belonging to an *R. pickettii* outbreak at the GUH in 1990 and thus adds to the number of nosocomial outbreaks caused by *R. mannitolytica*.

Differentiation of *R. mannitolytica* at the species and genus level

The distinctiveness of *R. mannitolytica* and *R. pickettii* biovar Va-1 and biovar Va-2 has been recognized previously. Although the study of King *et al.* (1979) was not conclusive and failed to establish '*P. thomasii*' as a valid species, Costas *et al.* (1990) concluded that SDS-PAGE of whole-cell proteins provided clear evidence that *R. pickettii* and '*P. thomasii*' were separate taxa, a finding confirmed by this study. On the other hand, Pan *et al.* (1992) reported that HPLC analysis of cell wall fatty acids could not distinguish *R. pickettii* biovar Va-1 from *R. pickettii* biovar 3/'*thomasii*', findings also confirmed by this study.

Several clear phenotypic differences exist between *R. mannitolytica* and the other *Ralstonia* species. *R. mannitolytica* can be differentiated from the other *Ralstonia* species, except for *Ralstonia* sp. strains LMG 19089 (RAL04/MC5) and LMG 19087 (RAL07/YL13), through its assimilation/acidification of mannitol and D-arabitol. *R. mannitolytica* strains differ from *R. pickettii* and *R. solanacearum* by their resistance towards desferrioxamine and from *R. pickettii* because of their lack of alkalization of tartrate and of nitrate reductase. The *R. pickettii* biovars Va-1 and Va-2 differ from each other by the lack of acid production from maltose and lactose for *R. pickettii* biovar Va-2 strains. The *R. solanacearum* strains tested could be differentiated from other species by acidification/assimilation of inositol and sucrose and lack of pyrrolidonyl arylamidase and of assimilation of caprate, malonate, propionate, suberate, acetate and lactate. *R. eutropha* strains were positive for alkaline phosphatase (Rosco) and assimilation of 3-hydroxybenzoate but did not alkalize mucate, in contrast to the other *Ralstonia* species. *R. paucula*

strains differed from *R. eutropha* and *R. gilardii* by strong urease production and from *R. eutropha* (no data available for *R. gilardii*) by alkalization of mucate but not of adipate and allantoin. Strains of *R. mannitolytica* were previously reported to be adonitol and ethanol acidification-negative, like the *R. pickettii* biovars Va-1 and Va-2, and cellobiose-positive, like *R. pickettii* biovar Va-1 (Pickett, 1994).

Yabuuchi *et al.* (1995) pointed to the difficulty of establishing reliable features to differentiate *Ralstonia* from *Burkholderia* at the generic level and stated that the three *Ralstonia* species (*R. pickettii*, *R. eutropha* and *R. solanacearum*) did not assimilate the carbohydrates galactose, mannitol, mannose or sorbitol, while the 11 *Burkholderia* species did. However, *R. mannitolytica* also assimilates and acidifies mannitol. As a consequence, differentiation between *R. mannitolytica* and *B. cepacia* genomovar II strains, which do not decarboxylate lysine or acidify sucrose (Vandamme *et al.*, 1997), is difficult, since *R. mannitolytica* and *B. cepacia* both produce acid from the same carbohydrates (with the exception of sucrose: 87% of *B. cepacia* strains are positive, whereas *R. mannitolytica* strains are negative) and since *R. mannitolytica* strains are unable to reduce nitrates, with *B. cepacia* strains unable to reduce nitrate to molecular nitrogen and with one-third of the *B. cepacia* strains reducing nitrate only to nitrite. Differentiation is only possible by means of a positive pyrrolidonyl arylamidase test for *R. mannitolytica*. Furthermore, in the routine clinical laboratory, *R. mannitolytica* can be differentiated from *P. fluorescens* and *Pseudomonas aeruginosa* by a negative pyoverdine test and from *P. fluorescens* by its inability to grow on *Salmonella*/*Shigella* agar and by a negative arginine dihydrolase test.

The Japanese environmental isolates LMG 19087 and LMG 19089 were phenotypically indistinguishable from each other, but according to 16S rDNA sequence and DNA-DNA hybridization studies they belong to two separate, as-yet undescribed *Ralstonia* species. LMG 19089 clusters most closely to *R. mannitolytica* according to its partially determined 16S rDNA sequence. In SDS-PAGE, it clusters close to *R. pickettii* biovar Va-1. Both strains can be differentiated from *R. mannitolytica* only by their ability to reduce nitrate and LMG 19087 also by lack of growth at 42 °C. LMG 19087 falls within the *R. pickettii* biovar Va-1 cluster according to its 16S rDNA sequence.

The Japanese environmental isolates LMG 19083 and LMG 19088 could be identified biochemically and according to 16S rDNA sequence and SDS-PAGE profile as genuine *R. pickettii* biovar Va-1 strains.

R. pickettii biovars Va-1 and Va-2 are easily distinguishable biochemically and some unambiguous 16S rDNA sequence differences are present, as shown in this study. DNA-DNA hybridization, as carried out here, separates the two representatives of each group tested, although Pickett & Greenwood (1980) found

84% DNA homology between a Va-1 strain and the type strain of *R. pickettii* (which is a Va-2 group strain) and Ralston *et al.* (1973) found 90% binding at 71 °C between Va-1 and Va-2 strains. In the SDS-PAGE clustering of whole-cell proteins, the *R. pickettii* strains seem somewhat heterogeneous, without clear-cut delineation between biovar Va-1 and biovar Va-2 strains.

Comparative 16S rDNA sequence analysis indicates that two sublineages may be discriminated within the genus *Ralstonia*, with the *R. eutropha* lineage on the one hand, which is composed of *R. eutropha*, *R. paucula* and *R. gilardii*, and the *R. pickettii* lineage on the other hand, with *R. pickettii*, *R. solanacearum*, *R. mannitolytica* and [*P.*] *syzygii*. This genotypic discrimination is supported by at least some phenotypic differences. Members of the *R. eutropha* lineage are colistin susceptible, do not produce acids from carbohydrates, assimilate oxalate but not galacturonate and have peritrichous flagella in contrast to members of the *R. pickettii* lineage, which are colistin resistant, have a polar flagellum and have biochemical characteristics distinct from those mentioned for the *R. eutropha* lineage. They also differ in viability on TSA at room temperature, with viability of at most 6 d for the *R. pickettii* lineage and at least 21 d for the *R. eutropha* lineage. Incomplete data are available for *R. gilardii*, which belongs to the *R. eutropha* lineage according to 16S rDNA sequence analysis, although it was reported to be monotrichous (Coenye *et al.*, 1999). Further studies, also including *R. gilardii* and *R. basileensis*, will indicate whether these findings can be confirmed.

Description of *Ralstonia mannitolytica* sp. nov.

Ralstonia mannitolytica (man.ni.to'ly.ti.ca. N.L. adj. *mannitolytica* cleaving mannitol).

The species accommodates the *Ralstonia pickettii* biovar 3/'*thomasii*' strains and at least some of the strains known as '*Pseudomonas thomasii*'. Cells are Gram-negative, non-sporulating rods that are motile by means of one polar flagellum (motility was not observed for the culture collection *R. mannitolytica* type strain, LMG 6866^T). Aerobic growth is observed at 30, 37 and 42 °C and strains are viable for less than 6 d on TSA at room temperature. Catalase and oxidase tests are positive. Nitrate and nitrite are not reduced. Strains are resistant to desferrioxamine, O:129 and colistin. No acid is produced from ethylene glycol. Urease, pyrrolidonyl arylamidase (Rosco), Tween esterase and phenylalanine deaminase tests are positive. Indole and hydrogen sulfide production, alkaline phosphatase (Rosco), arginine dihydrolase, lysine and ornithine decarboxylases, aesculin and gelatin hydrolysis tests are negative. Acid is produced oxidatively from glucose, L-arabinose, lactose, maltose, mannitol, D-arabitol and D-xylose. Alkalization occurs on minimal mineral agar with acetate, serine, malonate, β-alanine, 4-aminobutyrate, azelate, succinate, fumarate, butyrate, formate, malate, mucate, galacturonate, citrate, histidine and lactate, but not with

acetamide, adipate, alginate, allantoin, amygdalin, L-arginine, benzoate, L-ornithine, maleate or tartrate. Testing by means of API 20NE, API 50CH and ID 32GN indicates that the strains assimilate acetate, N-acetylglucosamine, L-alanine, L-arabinose, D-arabitol, 4-hydroxybenzoate, 3-hydroxybutyrate, caprate, citrate, fructose, galactose, gluconate, glucose, 2-ketogluconate, glycerol, histidine, D-lactate, malate, malonate, mannitol, L-proline, propionate, serine, suberate and D-xylose, but not adipate, amygdalin, L-fucose, glycogen, inositol, itaconate, 5-ketogluconate, maltose, mannose, melibiose, phenylacetate, rhamnose, ribose, sucrose, salicin, D-sorbitol or 3-hydroxybenzoate. Using the API ZYM system, the following enzymes are detected: C4 esterase, C8 esterase-lipase, C14 lipase, leucine arylamidase, acidic and alkaline phosphatase and phosphoamidase. Valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. The following cellular fatty acid components are present: 16:1^{9cis} (range 30–38 mol%), 16:0 (21–30 mol%), 18:1^{11cis} (7–15 mol%) and 14:0 (5–7 mol%). The DNA G+C content is 66.2 mol%.

The type strain is LMG 6866^T (deposited as '*Pseudomonas thomasii*' in 1972) (= NCIB 10805^T) (Phillips *et al.*, 1972). The *R. mannitolytica* strains used in this study have been deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Ghent, Belgium) as LMG 19090, LMG 19091 and LMG 19092.

ACKNOWLEDGEMENTS

This work was partly supported by the Concerted Research Action of the 'Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk onderzoek' (Belgium), grant no. 12050797. P.D.V. and M.V. are indebted to the Fund for Scientific Research for a position as Research Director and for research grants. Leen Van Simaey and Filippe Grillaert are acknowledged for excellent technical assistance. We also thank P. Green for strain information.

REFERENCES

- Baird, R. M., Elhag, K. M. & Shaw, E. J. (1976). *Pseudomonas thomasii* in a hospital distilled-water supply. *J Med Microbiol* **9**, 493–495.
- Brim, H., Heyndrickx, M., De Vos, P., Wilmotte, A., Springael, D., Schlegel, H. G. & Mergeay, M. (1998). Amplified rDNA restriction analysis and further genotypic characterisation of metal-resistant soil bacteria and related facultative hydro-geotrophs. *Syst Appl Microbiol* **22**, 258–268.
- Clark, W. A., Hollis, D. G., Weaver, R. E. & Riley, P. (1984). *Identification of Unusual Pathogenic Gram-negative Aerobic and Facultatively Anaerobic Bacteria*. Atlanta, GA: US Department of Health and Human Services, Public Health Service, Centers for Disease Control.
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes*

- faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.
- Costas, M., Holmes, B., Sloss, L. L. & Heard, S. (1990). Investigation of a pseudo-outbreak of '*Pseudomonas thomasi*' in a special-care baby unit by numerical analysis of SDS-PAGE protein patterns. *Epidemiol Infect* **105**, 127–137.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Dewhirst, F. E., Paster, B. J. & Bright, P. L. (1989). *Chromobacterium*, *Eikenella*, *Kingella*, *Neisseria*, *Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.). *Int J Syst Bacteriol* **39**, 258–266.
- Dimech, W. J., Hellyar, A. G., Kotiw, M., Marcon, D., Ellis, S. & Carson, M. (1993). Typing of strains from a single-source outbreak of *Pseudomonas pickettii*. *J Clin Microbiol* **31**, 3001–3006.
- Dowsett, E. (1972). Hospital infections caused by contaminated fluids. *Lancet* **i**, 1338.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fass, R. J. & Barnishan, J. (1976). Acute meningitis due to a *Pseudomonas*-like group Va-1 bacillus. *Ann Intern Med* **84**, 51–52.
- Fujita, S., Yoshida, T. & Matsubara, F. (1981). *Pseudomonas pickettii* bacteremia. *J Clin Microbiol* **13**, 781–782.
- Gardner, S. & Shulman, S. T. (1984). A nosocomial common source outbreak caused by *Pseudomonas pickettii*. *Pediatr Infect Dis J* **3**, 420–422.
- Gilligan, P. H. (1995). *Pseudomonas* and *Burkholderia*. In *Manual of Clinical Microbiology*, 6th edn, pp. 509–519. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Tenover. Washington, DC: American Society for Microbiology.
- Kahan, A., Philippon, A., Paul, G., Weber, S., Richard, C., Hazebrucq, G. & Degeorges, M. (1983). Nosocomial infections by chlorhexidine solution contaminated with *Pseudomonas pickettii* (biovar Va-1). *J Infect* **7**, 256–263.
- King, A., Holmes, B., Phillips, I. & Lapage, S. P. (1979). A taxonomic study of clinical isolates of *Pseudomonas pickettii*, '*P. thomasi*' and 'Group IVd' bacteria. *J Gen Microbiol* **114**, 137–147.
- Kodaka, H., Armfield, A. Y., Lombard, G. L. & Dowell, V. R., Jr. (1982). Practical procedure for demonstrating bacterial flagella. *J Clin Microbiol* **16**, 948–952.
- Lacey, S. & Want, S. V. (1991). *Pseudomonas pickettii* infections in a paediatric oncology unit. *J Hosp Infect* **17**, 45–51.
- Li, X., Del Dot, T., Dorsh, M., Stackebrandt, E., Sly, L. I. & Hayward, A. C. (1993). Phylogenetic studies on rRNA group II *Pseudomonas* using 16S rRNA sequences. *J Appl Bacteriol* **74**, 324–329.
- Lindsay, J. A. & Riley, T. V. (1991). Susceptibility to desferrioxamine: a new test for the identification of *Staphylococcus epidermidis*. *J Med Microbiol* **35**, 45–48.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Moissenet, D., Goujon, C. P., Garbarg-Chenon, A. & Vu-Thien, H. (1999). CDC group IVc-2: a new *Ralstonia* species close to *Ralstonia eutropha*. *J Clin Microbiol* **37**, 1777–1781.
- Osterhout, G. J., Valentine, J. L. & Dick, J. D. (1998). Phenotypic and genotypic characterization of clinical strains of CDC group IVc-2. *J Clin Microbiol* **36**, 2618–2622.
- Pan, H. J., Teng, L. J., Tzeng, M. S., Chang, S. C., Ho, S. W., Luh, K. T. & Hsieh, W. C. (1992). Identification and typing of *Pseudomonas pickettii* during an episode of nosocomial outbreak. *Chin J Microbiol Immunol* **25**, 115–123 (in Chinese).
- Phillips, I. & Eykyn, S. (1972). Contaminated drip fluids. *Brit Med J* **i**, 746.
- Phillips, I., Eykyn, S. & Laker, M. (1972). Outbreak of hospital infection caused by contaminated autoclaved fluids. *Lancet* **i**, 1258–1260.
- Pickett, M. J. (1994). Typing of strains from a single-source outbreak of *Pseudomonas pickettii*. *J Clin Microbiol* **32**, 1132–1133.
- Pickett, M. J. & Greenwood, J. R. (1980). A study of the Va-1 group of pseudomonads and its relationship to *Pseudomonas pickettii*. *J Gen Microbiol* **120**, 439–446.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Ralston, E., Palleroni, N. J. & Doudoroff, M. (1973). *Pseudomonas pickettii*, a new species of clinical origin related to *Pseudomonas solanacearum*. *Int J Syst Bacteriol* **23**, 15–19.
- Raveh, D., Simhon, A., Gimmon, Z., Sacks, T. & Shapiro, M. (1993). Infections caused by *Pseudomonas pickettii* in association with permanent indwelling intravenous devices: four cases and a review. *Clin Infect Dis* **17**, 877–880.
- Riley, P. S. & Weaver, R. E. (1975). Recognition of *Pseudomonas pickettii* in the clinical laboratory: biochemical characterization of 62 strains. *J Clin Microbiol* **1**, 61–64.
- Roberts, L. A., Collignon, P. J., Cramp, V. B., Alexander, S., McFarlane, A. E., Graham, E., Fuller, A., Sinickas, V. & Hellyar, A. (1990). An Australia-wide epidemic of *Pseudomonas pickettii* bacteraemia due to contaminated 'sterile' water for injection. *Med J Aust* **152**, 652–655.
- Seal, S. E., Jackson, L. A., Young, J. P. W. & Daniels, M. J. (1993). Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the Blood Disease Bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. *J Gen Microbiol* **139**, 1587–1594.
- Steinle, P., Stucki, G., Stettler, R. & Hanselmann, K. W. (1998). Aerobic mineralization of 2,6-dichlorophenol by *Ralstonia* sp. strain RK1. *Appl Environ Microbiol* **64**, 2566–2571.
- Suyama, T., Tokiwa, Y., Ouichanpagdee, P., Kanagawa, T. & Kamagata, Y. (1998). Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. *Appl Environ Microbiol* **64**, 5008–5011.
- Tatum, H. W., Ewing, W. H. & Weaver, R. E. (1974). Miscellaneous Gram-negative bacteria. In *Manual of Clinical Microbiology*, 2nd edn, pp. 270–294. Edited by E. H. Lennette, E. H. Spaulding & J. P. Truant. Washington, DC: American Society for Microbiology.
- Toschka, H. Y., Hopfl, P., Ludwig, W., Schleifer, K. H., Ulbrich, N.

- & Erdmann, V. A. (1988).** Complete nucleotide sequence of a 16S ribosomal RNA gene from *Pseudomonas aeruginosa*. *Nucleic Acids Res* **16**, 2348.
- Vandamme, P., Holmes, B., Vancanneyt, M. and 8 other authors (1997).** Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**, 1188–1200.
- Vandamme, P., Goris, J., Coenye, T., Hoste, B., Janssens, D., Kersters, K., De Vos, P. & Falsen, E. (1999).** Assignment of Centers for Disease Control group IVc-2 to the genus *Ralstonia* as *Ralstonia paucula* sp. nov. *Int J Syst Bacteriol* **49**, 663–669.
- Vauterin, L. & Vauterin, P. (1992).** Computer aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. *Eur J Microbiol* **1**, 37–41.
- Verschraegen, G., Claeys, G., Meeus, G. & Delanghe, M. (1985).** *Pseudomonas pickettii* as a cause of pseudobacteremia. *J Clin Microbiol* **21**, 278–279.
- Wauters, G., Van Bosterhaut, B., Janssens, M. & Verhaegen, J. (1998).** Identification of *Corynebacterium amycolatum* and other nonlipophilic fermentative corynebacteria of human origin. *J Clin Microbiol* **36**, 1430–1432.
- Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992).** Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251–1275.
- Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. & Nishiuchi, Y. (1995).** Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol Immunol* **39**, 897–904.
- Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G. J. & Woese, C. R. (1985).** Mitochondrial origins. *Proc Natl Acad Sci USA* **82**, 4443–4447.