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Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov.

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A polyphasic taxonomic study that included DNA–DNA hybridizations, DNA base ratio determinations, 16S rDNA sequence analysis, whole-cell protein and fatty acid analyses, AFLP (amplified fragment length polymorphism) fingerprinting and an extensive biochemical characterization was performed on 10 strains provisionally identified as *Alcaligenes faecalis*-like bacteria. The six environmental and four human isolates belonged to the genus *Ralstonia* and were assigned to a new species for which the name *Ralstonia gilardii* sp. nov. is proposed. The type strain is LMG 5886^T.

Keywords: *Ralstonia gilardii* sp. nov., *Ralstonia eutropha*, taxonomy

INTRODUCTION

The genus *Ralstonia* was established by Yabuuchi *et al.* (1995) to accommodate generically misnamed *Burkholderia* and *Alcaligenes* species: *Ralstonia pickettii* (the type species), *Ralstonia solanacearum* and *Ralstonia eutropha*. *Ralstonia* species occupy very diverse ecological niches. *R. pickettii* strains are isolated from various clinical sources (including urine, nasopharynx, wounds, blood and cerebrospinal fluid) (Riley & Weaver, 1975) and are responsible for nosocomial infections (McNeill *et al.*, 1985; Verschraegen *et al.*, 1985). *R. solanacearum* is one of the most important bacterial phytopathogenic species (Palleroni & Doudoroff, 1971), causing bacterial wilt on a wide range of crops, including potato, tomato, ginger and banana (Taghavi *et al.*, 1996). Finally, *R. eutropha* is an environmental organism isolated from sludge, soil and waste-water. Its ability to grow autotrophically has received special attention as a potential source of single-cell protein (Jenni *et al.*, 1988).

We performed a polyphasic taxonomic study to elucidate the taxonomic affiliation of 10 isolates from human clinical sources and the environment which phenotypically resembled *Alcaligenes faecalis*. Here we show that these isolates belong to a novel *Ralstonia* species for which we propose the name *Ralstonia gilardii* sp. nov.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. All of the strains were grown aerobically on Trypticase Soy Agar (BBL) and incubated at 37 °C.

Abbreviation: AFLP, amplified fragment length polymorphism.

The GenBank accession number for the fragment of the 16S rRNA gene of *Ralstonia gilardii* sp. nov. is AF076645.

Table 1. List of strains studied

API, Appareils et Procédés d'Identification, Montalieu-Vercieu, France; ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CDC, Center for Disease Control, Atlanta, GA, USA; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Sand Hutton, York, UK.

Species and strain designation	Other strain designation	Depositor	Source (if known)
R. pickettii			
LMG 5942^T	CCUG 3318^T	M. Pickett	Patient after tracheotomy (USA)
LMG 7001	CCUG 3314	M. Pickett	USA
LMG 7002	CCUG 3316	M. Pickett	USA
LMG 7004	CCUG 3319	M. Pickett	USA
LMG 7018	CCUG 3323	M. Pickett	USA
R. solanacearum			
LMG 2299^T	NCPPB 325^T	NCPPB	*Lycopersicon esculentum* (USA)
LMG 2300	NCPPB 339	NCPPB	*Solanum tuberosum* (Israel)
LMG 2303	NCPPB 789	NCPPB	*Musa* sp. (Honduras)
LMG 2304	NCPPB 792	NCPPB	*Tectona grandis* (Malaysia, 1959)
LMG 17145	NCPPB 1123	J. Van Vaerenbergh	*Lycopersicon esculentum* (New Guinea, 1961)
R. eutropha			
LMG 1190	ATCC 33178	M. Okazaki	Soil (Japan)
LMG 1196	Schlegel N9A	H. Schlegel	
LMG 1199^T	ATCC 17697^T	ATCC	Soil (USA, 1957)
LMG 1201	ATCC 17699	ATCC	Sludge (Germany, 1962)
LMG 1202	ATCC 17700	ATCC	Soil (USA, 1957)
R. gilardii			
LMG 3399	API 146-4-76	J. Gayral	Furuncle (USA)
LMG 3400	API 119-5-76	J. Gayral	Human cerebrospinal fluid (USA)
LMG 5886^T	API 141-2-84^T	D. Monget	Whirlpool
LMG 5887	API 142-2-84	D. Monget	Whirlpool
LMG 5888	API 143-2-84	D. Monget	Whirlpool
LMG 5910	API 188-2-84	D. Monget	Whirlpool
LMG 5913	API 191-2-84	D. Monget	Whirlpool
LMG 15537	CCUG 24719	G. L. Gilardi	Bone marrow (USA)
LMG 15540	CCUG 24724	G. L. Gilardi	Whirlpool drain (USA, 1984)
LMG 15541	CCUG 24725	G. L. Gilardi	Human cerebrospinal fluid

SDS-PAGE of whole-cell proteins. Preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). Strains were grown for 48 h at 37 °C. Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar 4.0 software package (Applied Maths).

Fatty acid methyl ester analysis. After an incubation period of 24 h, a loopful of well grown cells was harvested and fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as described by Vandamme *et al.* (1992).

DNA preparation. High-molecular-mass DNA for DNA–DNA hybridizations and determination of G+C content was prepared using a modified method of Marmur (1961) as described by Vandamme *et al.* (1992). Alternatively, small-scale DNA extracts were prepared as described by Pitcher *et al.* (1989).

AFLP (amplified fragment length polymorphism) fingerprinting. The preparation of template DNA for PCR was performed as described by Huys *et al.* (1996). One microgram of intact chromosomal DNA was digested with *Apa*I and *Taq*I (Pharmacia). After complete digestion, double-stranded restriction-halfsite specific adaptors were ligated to the restriction fragments with T4 ligase (Pharmacia). In the selective PCR amplification, primers B07 (5′ GACTGCGTACAGGCCCG 3′) and T11 (5′ GATGAGTCCTGACCGAG 3′) (selective bases at the 3′ end are underlined) were used. B07 was labelled at its 5′ end with a T4 kinase (Pharmacia) assay by using [γ-^{32}P]ATP (Amersham) as described by Vos *et al.* (1995). Electrophoresis, visualization of fragments, densitometric scanning and numerical analysis were performed as described by Huys *et al.* (1996).

16S rDNA sequencing. A fragment of the 16S rRNA gene (corresponding to positions 8–1541 in the *Escherichia coli* numbering system) of *R. gilardii* LMG 5886^T was amplified by PCR using conserved primers (5′ AGAGTTTGATCCTGGCTGAG 3′ and 5′ AAGGAGGTGATCCAGCCGCA 3′). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Sequencing was performed using an Applied Biosystems 377 DNA Sequencer and the protocols of the

Table 2. Sequencing primers

Name†	Synonym‡	Sequence (5′ → 3′)	Position§
16F358	*Gamma	CTC CTA CGG GAG GCA GCA GT	339–358
16F536	*PD	CAG CAG CCG CGG TAA TAC	519–536
16F926	*O	AAC TCA AAG GAA TTG ACG G	908–926
16F1112	*3	AGT CCC GCA ACG AGC GCA AC	1093–1112
16F1241	*R	GCT ACA CAC GTG CTA CAA TG	1222–1241
16R339	Gamma	ACT GCT GCC TCC CGT AGG AG	358–339
16R519	PD	GTA TTA CCG CGG CTG CTG	536–519
16R1093	3	GTT GCG CTC GTT GCG GGA CT	1112–1093

† F, Forward primer; R, reverse primer.

‡ Forward primers are indicated by '*'.

§ *E. coli* 16S rRNA gene sequence numbering.

manufacturer (Perkin-Elmer) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit. The sequencing primers used are listed in Table 2. Sequence assembly was performed by using the program Auto-Assembler (Perkin-Elmer). The closest related sequences were found using the FASTA program. Phylogenetic analysis was performed using the GeneCompar 2.1 software package (Applied Maths). The sequences of strains belonging to the same phylogenetic group were retrieved from the EMBL database and aligned with the consensus sequence. A phylogenetic tree was constructed based on the neighbour-joining method.

DNA–DNA hybridizations. The determination of the degree of DNA–DNA binding by using the initial renaturation rate method was performed as described by De Ley *et al.* (1970). Each value given is the mean of at least two hybridization experiments. The total DNA concentration was 0·059 mM and the optimal renaturation temperature in 2 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7·0) was 81 °C.

Determination of G+C content. Mean G+C values were determined by the thermal denaturation method and were calculated as described by De Ley (1970).

Phenotypic characterization. API microtest systems (API 50CH, API 50AO and API 50AA; bioMérieux) were used to analyse the assimilation of 147 organic compounds as sole carbon sources. The experimental procedure and the reading of the results of the auxanographic tests were performed as described previously (Kersters *et al.*, 1984). Classical phenotypic tests were performed as described previously (Vandamme *et al.*, 1993). API ZYM tests were performed according to the recommendations of the manufacturer (bioMérieux). Flagella staining was done using a solution of tannic acid (2 g), phenol (5%, w/v), $KAl(SO_4)_2$ (saturated) in 11 ml saturated crystal violet in 95% ethanol. Stained strains were viewed under a phase-contrast microscope.

Antimicrobial susceptibility testing. MIC values towards levofloxacin, ciprofloxacin, sparfloxacin, ofloxacin, HMR 3004, HMR 3647, erythromycin A, roxithromycin, clarithromycin and azithromycin were determined by using the agar dilution method conforming to National Committee for Clinical Laboratory Standards (1995) guidelines. Strains were grown on Mueller–Hinton agar (BRL) for 16–20 h at 35 °C.

RESULTS

SDS-PAGE of whole-cell proteins

Reproducibility was checked by preparing protein extracts in duplicate. The correlation level between the patterns obtained with different extracts of the same strain was more than 93% (data not shown). After numerical analysis and visual comparison of the profiles, five clusters could be delineated (Fig. 1). Cluster I comprises all *R. eutropha* strains investigated. Cluster II is composed of the *R. gilardii* strains. *R. solanacearum* LMG 2300 and LMG 2303 constitute cluster III. The *R. pickettii* strains investigated comprise cluster IV. *R. solanacearum* LMG 2304 and LMG 17145 constitute cluster V. The type strain of *R. solanacearum* occupied a separate position. Fig. 2 shows the whole-cell protein profiles of a selection of *R. gilardii*, *R. eutropha*, *R. pickettii* and *R. solanacearum* strains.

Cellular fatty acid analysis

The cellular fatty acid composition of *R. gilardii* strains and of the type strains of *R. pickettii*, *R. solanacearum* and *R. eutropha* was determined (Table 3). In addition, the cellular fatty acid composition of the *A. faecalis* type strain LMG 1229^T was available for comparison (P. Vandamme & M. Vancanneyt, unpublished data). Both quantitative and qualitative differences occur between the taxa examined. The *R. gilardii* strains form a homogeneous group and can easily be distinguished from the other organisms examined, with 17:0 cyclo, 16:0 2-OH and 19:0 cyclo $\omega 8c$ as most important differential characters.

AFLP fingerprinting

After selective amplification of template DNA with primers B07 and T11, 40–60 PCR products could be visualized. Reproducibility was checked by preparing PCR products in duplicate and was always higher than 90% (data not shown). After numerical analysis, four

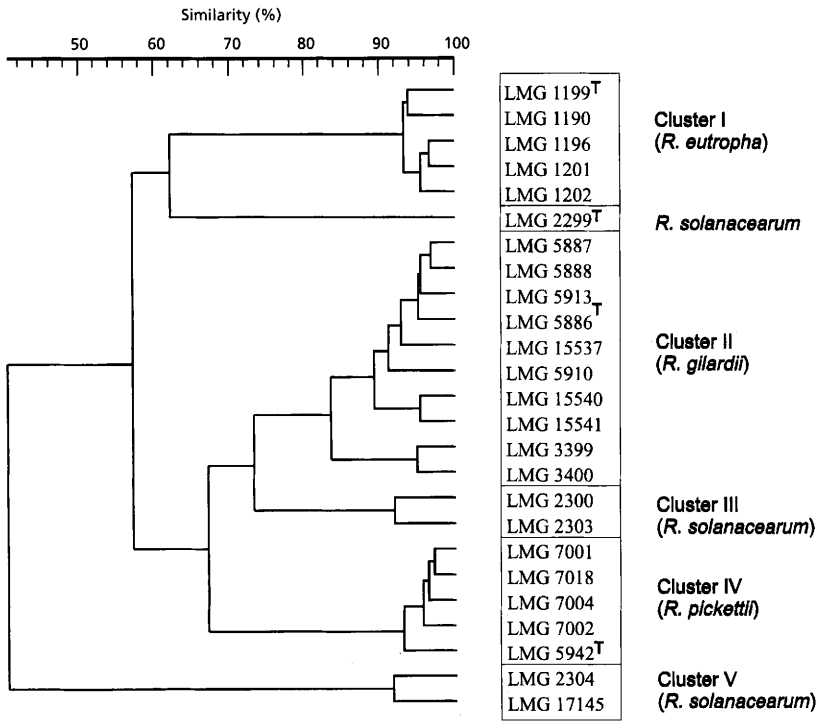

Fig. 1. Dendrogram derived from the unweighted pair group mean linkage of correlation coefficients between the protein patterns of the strains studied. The correlation coefficient is expressed as percentage similarity for convenience.

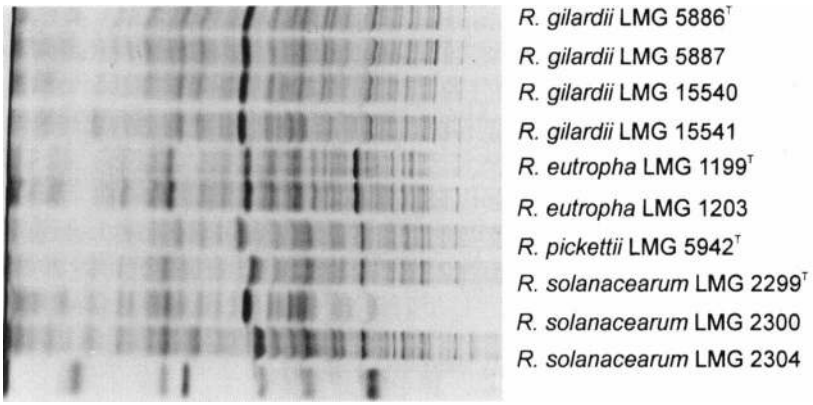

Fig. 2. Electrophoretic protein patterns of a selection of strains investigated. The molecular mass markers used (lane at the bottom) were (from left to right) lysozyme (14500 Da), trypsin inhibitor (20000), trypsinogen (24000), glyceraldehyde-3-phosphate dehydrogenase (36000), egg albumin (45000), bovine albumin (60000) and β-galactosidase (116000).

Table 3. Fatty acid composition of the strains studied

Data are expressed as a percentage. Those fatty acids for which the mean amount for all taxa was less than 1 % are not given. Mean percentages ± SD are given for *R. gilardii*. TR, Trace amount (less than 1 %); ND, not detected. Summed feature 1 comprises 14:1ω5*c*, 14:1ω5*t* or both. Summed feature 3 comprises 14:0 3-OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10·928, 12:0 ALDE or any combination of these fatty acids. Summed feature 7 comprises 18:1ω7*c*, 18:1ω9*t*, 18:1ω12*t* or any combination of these fatty acids.

Strain	12:0	14:0	16:0	17:0 cyclo	18:0	16:1ω7*c*	16:1 ω5*c*	16:1 2-OH	16:0 2-OH	16:0 3-OH	19:0 cyclo ω8*c*	18:1 2-OH	Summed feature 1	Summed feature 3	Summed feature 7
R. solanacearum LMG 2299T	TR	36·8	6·8	ND	TR	ND	TR	ND	ND	2·2	ND	ND	10·7	41·6	ND
R. eutropha LMG 1199T	4·4	10·8	25·2	ND	TR	41·4	TR	ND	ND	1·2	ND	ND	2·7	12·4	1·5
R. pickettii LMG 5942T	ND	5·5	28·8	2·7	TR	27·0	TR	2·7	TR	ND	TR	3·1	ND	7·7	17·9
R. gilardii (10 strains)	ND	4·7 ± 0·4	24·4 ± 1·3	8·9 ± 3·4	1·3 ± 0·1	20·9 ± 3·2	ND	ND	2·1 ± 0·2	ND	3·4 ± 1·8	ND	ND	7·3 ± 0·7	25·7 ± 2·4
A. faecalis LMG 1229T	2·1	1·4	36·8	28·8	TR	ND	ND	ND	ND	TR	TR	ND	ND	10·4	5·7

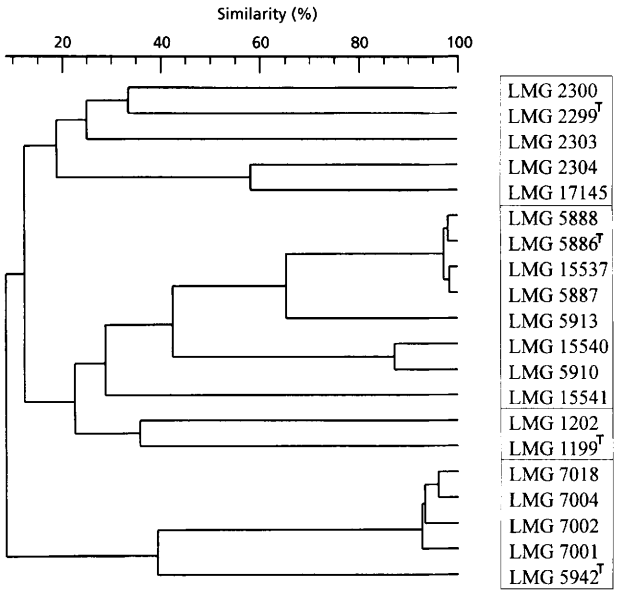

Fig. 3. Dendrogram derived from the unweighted pair group mean linkage of correlation coefficients between the AFLP patterns of the strains studied. The correlation coefficient is expressed as percentage similarity for convenience.

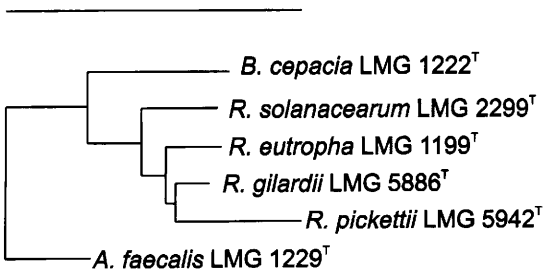

Fig. 4. Distance matrix tree showing the phylogenetic relationships of *Ralstonia* species based on 16S rDNA sequence comparisons. *Burkholderia cepacia* and *A. faecalis* were used as outgroups in this analysis. Bar, 10% sequence dissimilarity.

clusters could be delineated (Fig. 3). Cluster I is composed of the *R. solanacearum* strains investigated, cluster II is composed of *R. gilardii* strains, cluster III is composed of the two *R. eutropha* strains investigated and cluster IV is composed of *R. pickettii* strains.

16S rDNA sequencing

The 16S rRNA gene of *R. gilardii* LMG 5886^T shows 97·95% sequence similarity to the 16S rRNA gene of *R. eutropha* LMG 1199^T (M32021). The similarity to the *R. solanacearum* LMG 2299^T (X67036) and *R. pickettii* LMG 5942^T (X67042) 16S rRNA genes is 96·16 and 95·48%, respectively. The similarity to

Table 4. Range of MIC values and the MIC for 50 and 90% of the *R. gilardii* strains investigated

Strains investigated: LMG 5886^T, LMG 5887, LMG 5888, LMG 5910, LMG 5913, LMG 15537 and LMG 15541.

Antibiotic	Range (μg ml^{-1})	MIC_{50} (μg ml^{-1})	MIC_{90} (μg ml^{-1})
Levofloxacin	0·25–4	1	4
Ciprofloxacin	0·25–4	1	4
Ofloxacin	1–8	2	8
Sparfloxacin	0·03–2	0·06	2
HMR 3004	2–4	2	4
HMR 3647	1–4	2	4
Erythromycin A	4–16	8	16
Roxithromycin	8–32	16	32
Clarithromycin	4–16	8	16
Azithromycin	0·5–2	1	2

Table 5. Phenotypic characteristics useful for the differentiation of *R. gilardii* from other *Ralstonia* species and from *A. faecalis*

Assimilation tests were performed on LMG 5886^T, LMG 5887, LMG 5888, LMG 5910 and LMG 5913; other characteristics were tested on these and three additional strains (LMG 15537, LMG 15540 and LMG 15541) unless specified otherwise. Flagellation was tested on *R. gilardii* LMG 5886^T, LMG 5910 and LMG 15540; data for the other species are from Yabuuchi *et al.* (1995). +, More than 90% of strains gave a positive reaction; −, fewer than 10% of strains gave a positive reaction; D+, between 10 and 90% of strains gave a positive reaction and the type strain was positive; D−, between 10 and 90% of strains gave a positive reaction and the type strain was negative; V, between 11 and 79% of strains gave a positive reaction.

Characteristic	*R. gilardii*	*R. eutropha**	*R. pickettii**	*R. solanacearum**	*A. faecalis*†
Catalase activity	+	+	−	+	+
Growth at 42 °C	+	+	+	−	+
Growth on 10% lactose	−	+	+	+	−
Nitrate reduction	−	+	+	+	−
Nitrite reduction	−	+	+	+	+
Denitrification	−	−	+	+	−
Urease activity	−	−	+	+	−
Growth on 0·5% NaCl	+	+	+	−	+
Flagellation	One, polar	One–five, peritrich	One, polar	None	Peritrich
Assimilation of:					
D-Glucose	−	−	+	+	−
D-Galactose	−	−	+	D−	−
p-Hydroxybenzoate	−	D−	+	D−	−
D-Fructose	−	+	+	D+	−
2-Ketogluconate	−	D+	+	−	−
D-Malate	+	+	−	−	−
L-Arabinose	−	−	+	−	−
Glucosamine	−	−	+	−	−
Aconitate	−	D−	+	−	V
n-Caproate	−	+	+	−	V
L-Cysteine	−	D−	+	D+	V
D-Fucose	−	−	+	−	−
Mesaconate	+	+	−	−	−
Itaconate	+	+	−	−	−
D-Xylose	−	−	+	−	−
L-Tartrate	−	−	+	D−	−
N-Acetylglucosamine	−	+	+	−	−
DL-Norleucine	−	+	−	−	+

* Data for assimilation tests from Gillis *et al.* (1995).

† Data from Kersters & De Ley (1984).

Burkholderia cepacia LMG 1222^T (M22518), *A. faecalis* LMG 1229^T (D88008) and other taxa is below 94%. A dendrogram showing the phylogenetic position of *R. gilardii* is shown in Fig. 4.

DNA–DNA hybridizations

The level of DNA–DNA binding between *R. gilardii* LMG 5886^T and LMG 5910 was 100%; 0% was found between *R. gilardii* LMG 5886^T and *R. eutropha* LMG 1199^T.

Determination of G+C content

The G+C content of strains LMG 5886^T and LMG 5910 is 68·3 and 68·1 mol%, respectively.

Antimicrobial susceptibility testing

The range of MIC values and the MIC_{50} and MIC_{90} of the strains are given in Table 4.

Phenotypic characterization

The assimilation of carbon substrates, using the microtest systems API 50CH, API 50AO and API 50AA, was examined for five *R. gilardii* strains: LMG 5886^T, LMG 5887, LMG 5888, LMG 5910 and LMG 5913. All strains investigated assimilated pyruvate, succinate, glycerol, L-α-alanine, acetate, DL-lactate, L-histidine, L-tyrosine, DL-glycerate, propionate, L-valine, L-proline, L-serine, L-phenylalanine, DL-3-hydroxybutyrate, D-malate, isobutyrate, butyrate, L-

isoleucine, L-leucine, L-tryptophan, β-alanine, *n*-valerate, levulinate, pelargonate, caprate, 2-aminobenzoate, azelate, sebacate, glutarate, DL-3-aminobutyrate, pimelate, citraconate, mesaconate, itaconate, isovalerate, sucrose, *meso*-tartrate, gluconate, fumarate, L-malate, L-aspartate and L-glutamate. None of the strains examined assimilated D-glucose, inositol, D-galactose, sorbitol, mannitol, trehalose, D-mannose, DL-glycerate, *p*-hydroxybenzoate, DL-4-aminobutyrate, betaine, citrate, 2-ketogluconate, D-fructose, D-arabitol, L-arabinose, D-lyxose, glucosamine, aconitate, L-arginine, *N*-acetylglucosamine, sarcosine, L-ornithine, L-lysine, DL-kynurenine, *n*-caproate, heptanoate, DL-5-aminovalerate, ethanolamine, L-cysteine, dulcitol, D-arabinose, D-tagatose, D-fucose, amygdalin, ribose, malonate, 2-ketoglutarate, DL-2-aminobutyrate, L-citrulline, amylamine, arbutin, salicin, diaminobutane, L-rhamnose, 5-ketogluconate, xylitol, L-arabitol, *m*-hydroxybenzoate, butylamine, tryptamine, adonitol, ethylamine, erythritol, oxalate, L-methionine, maleate, D-tartrate, terephthalate, lactose, D-xylose, L-xylose, D-tryptophan, L-mandelate, methyl D-glucoside, D-turanose, *o*-hydroxybenzoate, glycine, urea, acetamide, D-melibiose, L-tartrate, benzoate, D-cellobiose, benzylamine, histamine, β-gentiobiose, D-raffinose, spermine, maltose, methyl D-mannoside, D-melezitose, starch, D-mandelate, trigonellin, glycogen, creatine, 3-aminobenzoate, 4-aminobenzoate, phthalate, inulin, methylxyloside, L-sorbose, aesculin or isophthalate.

Classical phenotypic tests and API ZYM tests were performed on eight *R. gilardii* strains (including the type strain), five *R. pickettii* strains, five *R. solanacearum* strains and the type strain of *R. eutropha*. The following features are present in all of the *R. gilardii* strains investigated: oxidase and catalase activity; growth at 30, 37 and 42 °C; growth on Drigalski agar; growth in 0·5 and 1·5% NaCl; alkaline phosphatase, C_4-esterase, C_8-esterase and leucine-arylamidase. The following features were absent in all of the *R. gilardii* strains investigated: haemolysis and odour on horse blood; fermentation of D-glucose, adonitol, D-fructose and D-xylose; fluorescence on King's B medium; growth on cetrimide agar, 10% lactose, 4·5 and 6% NaCl; Tween 80 hydrolysis; amylase and urease activity; nitrate and nitrite reduction; denitrification; liquefaction of gelatine; β-galactosidase and DNase activity; indole production; growth on acetamide; production of acid or H_2S from TSI; C_{14}-lipase, cysteine arylamidase, valine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, tryptophanase and arginine dihydrolase activity. The following features were strain-dependent: tolerance to penicillin (10 μg per disc); motility at 30 °C and in O/F medium; growth on 3·0% NaCl and assimilation of D-α-alanine, L-threonine, DL-norvaline, L-norleucine, caprylate, glycolate, maltose, caprate, adipate, citrate and phenylacetate.

Characteristics useful for the differentiation of *R. gilardii* from other *Ralstonia* species are given in Table 5. Other data are not shown.

DISCUSSION

We performed a polyphasic taxonomic study to identify 10 strains phenotypically resembling *A. faecalis* (like *A. faecalis*, the strains give negative reactions in a large number of phenotypic tests). Six strains were isolated from whirlpools and four from human clinical samples. The results of this study allowed us to assign all 10 strains to a new species, for which we propose the name *R. gilardii* sp. nov.

Taxonomic position of *R. gilardii*

Comparison of the 16S rDNA sequence of strain LMG 5886^T with those of other species of the β-*Proteobacteria* indicated that its closest relative is *R. eutropha* (97·95% sequence similarity) (Fig. 4). However, DNA–DNA hybridization between LMG 5886^T and the *R. eutropha* type strain revealed no significant binding. DNA–DNA hybridizations between strains of other species were not performed as the similarity level between their respective rRNA genes was below 97% (Stackebrandt & Goebel, 1994). These data unambiguously indicated that this taxon represents a novel *Ralstonia* species. Its DNA base ratio of 68–69% is also within the range of the genus *Ralstonia* (65–70%; Yabuuchi *et al.*, 1995).

Differentiation of *R. gilardii* from other *Ralstonia* species and from *A. faecalis*

Both whole-cell protein and fatty acid analyses indicate that the *R. gilardii* strains form a homogeneous group which can easily be differentiated from other *Ralstonia* species (Figs 1 and 2, Table 3). The finding that the *R. solanacearum* strains investigated can be found in two clusters and that the type strain occupied a separate position is remarkable. This heterogeneity is also obvious in Fig. 2.

AFLP is a genomic fingerprinting technique based on the selective amplification of restriction fragments from a total digest of genomic DNA. It has been reported that this method allowed differentiation of *Xanthomonas* species (Janssen *et al.*, 1996), *Aeromonas* species (Huys *et al.*, 1996; Janssen *et al.*, 1996) and *Acinetobacter* species (Janssen *et al.*, 1997) and that its results are generally consistent with DNA–DNA hybridization levels. In this study, all *Ralstonia* species were easily distinguished (Fig. 3) and the results were in agreement with results obtained by DNA–DNA hybridization (Ralston *et al.*, 1973; this study). The results obtained were also in agreement with 16S rRNA sequence analysis, which indicated that *R. gilardii* is most closely related to *R. eutropha*. Interestingly, the low mean linkage level of *R. solanacearum*

strains (cluster I, 18·7±3·6%) confirms that this species is rather heterogeneous, as reported by Palleroni & Doudoroff (1971).

Using biochemical tests, *R. gilardii* can be differentiated from *R. eutropha* by its inability to assimilate D-fructose, *n*-caproate, *N*-acetylglucosamine and DL-norleucine, and by the absence of nitrate and nitrite reduction and growth on 10% lactose. *R. gilardii* can be differentiated from *R. solanacearum* by the assimilation of D-malate, mesaconate and itaconate by the former and D-glucose by the latter. *R. gilardii* can easily be differentiated from *R. pickettii* since the latter is catalase-negative, reduces nitrate and nitrite and is capable of denitrification. Additional characteristics useful for the differentiation of *R. gilardii* from other *Ralstonia* species are given in Table 5.

In addition, *R. gilardii* can be differentiated from *A. faecalis* by the absence of nitrite reduction and assimilation of DL-norleucine, and by the assimilation of D-malate, mesaconate and itaconate (Kersters & De Ley, 1984). Differentiation of *R. gilardii* and *A. faecalis* is also possible based on their cellular fatty acid composition, with the presence of 12:0 and absence of 18:0, 16:1ω7*c* and 19:0 cyclo ω8*c* fatty acids being characteristic for *A. faecalis*. In addition, the G+C content of *A. faecalis* (55·9–59·4 mol%) is much lower than the G+C content of *R. gilardii* (Kersters & De Ley, 1984).

Description of *Ralstonia gilardii* sp. nov.

Ralstonia gilardii (gi.lar'di.i. M.L. gen. n. *gilardii* in honour of G. L. Gilardi, an American microbiologist who contributed much to our knowledge of *Alcaligenes* species).

R. gilardii cells are Gram-negative, non-sporulating rods that are motile by means of one polar flagellum. Growth is observed at 30, 37 and 42 °C. Catalase and oxidase activities are present. Nitrate and nitrite are not reduced. No denitrification. No urease, β-galactosidase and DNase activity. No liquefaction of gelatin. No aesculin hydrolysis. No indole production. Additional characteristics are listed in Results above. Characteristics differentiating *R. gilardii* from related taxa are summarized in Table 5. The following fatty acid components are present: 14:0, 16:0, 17:0 cyclo, 18:0, 16:1ω7*c*, 16:0 2-OH, 19:0 cyclo ω8*c*, summed feature 3 and summed feature 7. G+C content is between 68 and 69 mol%. Type strain is LMG 5886^T (= API 141-2-84^T), isolated from a whirlpool. Phenotypic characteristics of the type strain are the same as described above for the species. G+C content is 68·3 mol%. In addition, the type strain does not assimilate adipate, citrate, L-norleucine, DL-norvaline and caprylate but does assimilate caprate, phenylacetate, glycolate, D-α-alanine and L-threonine. All *R. gilardii* strains have been deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Gent, Gent, Belgium) and the CCUG (Culture Collection, University of Göteborg, Göteborg, Sweden).

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