Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend.

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Thirty-one heavy-metal-resistant bacteria isolated from industrial biotopes were subjected to polyphasic characterization, including 16S rDNA sequence analysis, DNA–DNA hybridizations, biochemical tests, whole-cell protein and fatty-acid analyses. All strains were shown to belong to the *Ralstonia* branch of the β -Proteobacteria. Whole-cell protein profiles and DNA–DNA hybridizations revealed two clearly distinct groups, showing low similarity to known *Ralstonia* species. These two groups, of 8 and 17 isolates, were assigned to two new species, for which the names *Ralstonia campinensis* sp. nov. and *Ralstonia metallidurans* sp. nov. are proposed. The type strains are WS2^T (= LMG 19282^T = CCUG 44526^T) and CH34^T (= LMG 1195^T = DSM 2839^T), respectively. Six isolates were allocated to *Ralstonia basilensis*, which presently contains only the type strain; an emendation of the latter species description is therefore proposed.

Keywords: *Ralstonia*, metal-resistance, taxonomy, industrial biotopes

INTRODUCTION

Metal resistance has been reported for a number of Gram-negative bacteria belonging to the *Ralstonia* lineage of the β -Proteobacteria (Diels & Mergeay, 1990; Mergeay *et al.*, 1985; Schmidt *et al.*, 1991; Schmidt & Schlegel, 1994). Resistance to Cd²⁺, Zn²⁺ and Co²⁺ has been shown to be located on a *czc* operon of the plasmid pMOL30 (240 kb), while Co²⁺ combined with Ni²⁺ resistance is located on the *cnr* operon of the plasmid pMOL28 (163 kb) (Liesegang *et al.*, 1993; Mergeay *et al.*, 1985; Nies *et al.*, 1987; Siddiqui *et al.*, 1989). Other plasmid-borne resistances to metals include resistance to copper (*cop*), lead (*pbr*) and mercury (*mer*) on pMOL30 and resistance to chromate (*chr*) and mercury on pMOL28 (for a review see Mergeay, 2000). Hybridization with a *czc* gene

probe showed the spread of the former resistance types over a variety of closely related taxa (Brim *et al.*, 1999).

The application of metal-resistant bacteria in bioremediation processes offers attractive perspectives for biomonitoring (via biosensors), treatment of wastewater and the recycling of polluted soils (Collard *et al.*, 1994; Diels *et al.*, 1995). In the case of the *Ralstonia* strains mentioned above, knowledge of their exact taxonomic position is of the utmost importance. Indeed, in addition to organisms that can be used for bioremediation of polluted soils, the genus *Ralstonia* also contains an important plant pathogen (*Ralstonia solanacearum*; Hayward, 1991) and several opportunistic human pathogens such as *Ralstonia pickettii* (Ralston *et al.*, 1973), *Ralstonia mannitolitica* (De Baere *et al.*, 2001), *Ralstonia gilardii* (Coenye *et al.*, 1999) and *Ralstonia paucula* (Vandamme *et al.*, 1999). *Ralstonia* strains with potential large-scale applications for bioremediation should obviously be distinct from (opportunistic) pathogens of humans and plants. Recent taxonomic studies describing novel *Ralstonia* species highlight not only the unsuspected

The EMBL accession numbers for the 16S rRNA gene sequences of *R. campinensis* WS2^T and *R. basilensis* ER121 and DSM 11853^T are AF312020, AF312021 and AF312022, respectively.

Table 1. List of strains studied

Culture collections are abbreviated as: API, Appareils et Procédés d'Identification, Montalieu-Vercieu, France; ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Sweden; CDC, Center for Disease Control, Atlanta, GA, USA; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, UK.

Strain number as received	Other strain designation(s)	Source
<i>Ralstonia campinensis</i>		
DS185	LMG 19284	Zinc desert, Lommel, Belgium
LH103	LMG 19285	Non-ferrous industry, Tienen, Belgium
LH104		Non-ferrous industry, Tienen, Belgium
LH105	LMG 19283	Non-ferrous industry, Tienen, Belgium
LH106		
WS2 ^T	LMG 19282 ^T , CCUG 44526 ^T	Zinc desert, Lommel, Belgium
WS3		
WS5		Zinc desert, Lommel, Belgium
<i>Ralstonia eutropha</i>		
ATCC 17697 ^T	LMG 1199 ^T , TF93 ^T	Soil (USA, 1957)
ATCC 17698	LMG 1200	Garden-pond sludge, Germany
ATCC 17699	LMG 1201, H16	Sludge, Weende-Quelle, Göttingen, Germany
ATCC 17700	LMG 1202, H20	Soil, USA
ATCC 17702	LMG 1203	Soil
ATCC 17704	LMG 1204	Soil
ATCC 17705	LMG 1205	Soil
ATCC 17706	LMG 1206	
ATCC 17709	LMG 1209	Soil, USA
ATCC 17710	LMG 1210	USA
ATCC 25207	LMG 1211	
ATCC 33178	LMG 1190	Paddy-field soil, Japan
N9A	LMG 1196	
<i>Ralstonia basilensis</i>		
DSM 11853 ^T	LMG 18990 ^T , LMG 19474 ^T , RK1 ^T	Freshwater-pond sediment, Amponville, France
ER8	LMG 19287	Non-ferrous industry, Overpelt, Belgium
ER107		Non-ferrous industry, Overpelt, Belgium
ER121		Non-ferrous industry, Overpelt, Belgium
ER122	LMG 19286	Non-ferrous industry, Overpelt, Belgium
KS1	LMG 19289	Zinc desert, Lommel, Belgium
SV884	LMG 19288	
<i>Ralstonia metallidurans</i>		
31A	LMG 19293	Metal factory, Holzminden, Germany
AB2		Mine area, Kipushi, Congo
AS2	LMG 19295	Mine area, Likasi-Sud, Congo
AS167	LMG 19291	Mine area, Likasi-Sud, Congo
AS168		Mine area, Likasi-Sud, Congo
CH34 ^T	LMG 1195 ^T , CCUG 38400 ^T , DSM 2839 ^T	Wastewater, 'Métallurgie de Prayon' zinc factory, Liège, Belgium
CH42		Polluted sediments, 'Métallurgie de Prayon' zinc factory, Liège, Belgium
CH45		Polluted sediments, 'Métallurgie de Prayon' zinc factory, Liège, Belgium
CH79	LMG 19296	Polluted sediments, 'Métallurgie de Prayon' zinc factory, Liège, Belgium
Sh2-1	LMG 19294	Mine area, Shituru, Congo
SV661	LMG 19290	Non-ferrous industry, Beerse, Belgium
VA1		Mine area, Likasi-Sud, Congo
VA2		Mine area, Likasi-Sud, Congo
VA3		Mine area, Likasi-Sud, Congo
VA7	LMG 19297	Mine area, Likasi-Sud, Congo
VA10	LMG 19292	Mine area, Likasi-Sud, Congo
VA11		Mine area, Likasi-Sud, Congo
<i>Ralstonia pickettii</i> CCUG 3318 ^T	LMG 5942 ^T , ATCC 27511 ^T	Patient after tracheotomy, Los Angeles, USA
<i>Ralstonia solanacearum</i> NCPPB 325 ^T	LMG 2299 ^T , ATCC 11696 ^T	<i>Lycopersicon esculentum</i> (tomato), USA
<i>Ralstonia gilardii</i> API 141-2-84 ^T	LMG 5886 ^T	Whirlpool
<i>Ralstonia paucula</i> CDC E6793 ^T	LMG 3244 ^T	Human, respiratory tract, USA
<i>Ralstonia paucula</i> CIP 62.31	LMG 3413, CCUG 13723	
<i>Ralstonia oxalatica</i> Oxl ^T	LMG 2235 ^T , DSM 1105 ^T	Indian earthworm
<i>Ralstonia mannitolitica</i> NCIB 10805 ^T	LMG 6866 ^T	Hospital outbreak, St Thomas' Hospital, London, UK
<i>Ralstonia</i> sp. ATCC 17707	LMG 1207	Soil
<i>Ralstonia</i> sp. JMP134	LMG 1197	Agricultural soil, Australia

biodiversity of the genus but also its weak and incomplete internal classification. The present report deals with the polyphasic classification of 31 metal-resistant strains, isolated from diverse industrial locations. Six strains could be allocated to *Ralstonia*

basilensis, while two groups, of 8 and 17 strains, respectively, are representatives of new *Ralstonia* species, for which we propose the names *Ralstonia campinensis* sp. nov. and *Ralstonia metallidurans* sp. nov.

Fig. 1. Dendrogram based on UPGMA clustering of SDS-PAGE analyses of whole-cell proteins of *Ralstonia* isolates. The zones used for numerical analysis were between 18.6 and 32.1 kDa and 38 and 107 kDa. Similarities are expressed as percentages.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. After being checked for purity, subcultures of all strains were maintained on slants of Tryptic soy agar [TSA; Trypticase soy broth (Becton & Dickinson) supplemented with 15 g Bacto Agar (Difco) l⁻¹] in the refrigerator and lyophilized for long-term storage.

PAGE of whole-cell proteins. Cells were grown for 48 h on TSA at 37 °C. SDS protein extracts were prepared and separated by electrophoresis as described by Pot *et al.* (1994). The collected data were interpreted as described by Vauterin & Vauterin (1992), using the GELCOMP 4.2 software (Applied Maths). Similarity between all pairs of electrophoresis patterns was calculated by Pearson's product-moment correlation coefficient, expressed as a percentage.

DNA-DNA hybridizations. DNA was prepared by the method of Marmur (1961), as modified by Vandamme *et al.* (1992), or by a modification of the protocol of Pitcher *et al.* (1989). Briefly, 500–750 mg cell mass was harvested from agar

surfaces, washed with 15 ml RS buffer (0.15 M NaCl, 0.01 M EDTA; pH 8.0), centrifuged and resuspended in 3 ml TE buffer (1.21 g Tris/HCl, 2 ml 0.5 M EDTA; pH 8.0 in 1 l MilliQ water). Fifteen ml GES reagent (600 g guanidinium thiocyanate, 200 ml 0.5 M EDTA; pH 8.0, 10 g Sarcosyl in 1 l MilliQ water) was added and the suspension was mixed gently until cell lysis occurred and then left on ice for 15 min. Ammonium acetate (7.5 ml, 7.5 M) was added and the suspension was mixed again and left on ice for 15 min. Ten ml chloroform/isoamyl alcohol (24:1) was added and the suspension was mixed vigorously. After 20 min of centrifugation at 17000 g, the upper phase was transferred to a disposable plastic beaker and 0.54 vols 2-propanol were added. The DNA was spooled onto a glass rod, washed in a series of ethanol solutions (70, 80 and 90% v/v), air-dried and dissolved overnight in 5 ml TE buffer. When the DNA was dissolved completely, 25 µl RNase solution (2 mg ml⁻¹) was added and incubated for 1 h at 37 °C. After RNase treatment, 625 µl acetate/EDTA (3 M sodium acetate, 1 mM EDTA; pH 7.0) was added and the chloroform extraction repeated. The supernatant was again transferred to a disposable plastic beaker and DNA was precipitated with 2 vols ethanol, spooled onto a glass rod, washed in an

Fig. 2. Photograph of whole-cell proteins patterns of *Ralstonia* strains illustrating the presence of reproducible strain-specific distortions. The molecular mass markers (M) used were (from left to right) lysozyme (14.5 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and β -galactosidase (116 kDa).

ethanol series and air-dried. Finally, the DNA was dissolved in 1 ml $0.1 \times$ SSC ($1 \times$ SSC contains 0.15 M NaCl and 0.015 M sodium citrate).

DNA–DNA hybridizations were performed either by the initial renaturation rate method of De Ley *et al.* (1970) or with the microplate method as described by Ezaki *et al.* (1989). In the latter case, an HTS7000 Bio Assay Reader (Perkin Elmer) was used for fluorescence measurements in white MaxiSorp (Nunc) microplates. The hybridization conditions were respectively 81 °C in $2 \times$ SSC and 50 °C in $2 \times$ SSC with 50% (v/v) formamide for the initial renaturation method and the microplate method. Results obtained by the two methods were shown previously to correlate very well (Goris *et al.*, 1998).

DNA base compositions. DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The analysis parameters were as reported in Logan *et al.* (2000).

16S rDNA sequencing. 16S rDNA sequencing was performed using an Applied Biosystems 377 DNA sequencer as described by Coenye *et al.* (1999). Partial sequences were assembled using the program AUTO ASSEMBLER (Applied Biosystems) and phylogenetic analysis was performed using the BioNUMERICS 1.5 software package (Applied Maths).

GC analysis of methylated fatty acids. Cells were grown for 24 h on TSA at 28 °C and fatty acid methyl esters were extracted, prepared, separated and identified by using the Microbial Identification System (Microbial ID) as described before (Vauterin *et al.*, 1991).

Phenotypic tests. Classical phenotypic tests were performed as described previously (De Vos *et al.*, 1985). API 20 NE and API ZYM (bioMérieux) were utilized according to the protocol supplied by the manufacturer.

RESULTS

PAGE of whole-cell proteins

The result of a numerical analysis based on whole-cell protein profiles is shown in Fig. 1. A zone corresponding to molecular masses between 32.1 and

38 kDa was omitted from the analysis. This zone comprised a distorted band in the profile of some cluster I (*R. campinensis*) strains, e.g. WS2^T (see Fig. 2). Six (I–VI) major clusters and 12 strains holding a distinct position in the dendrogram (Fig. 1) were differentiated above a correlation level of 85%. The *Ralstonia eutropha* reference strains formed a single homogeneous cluster (cluster III), grouping above 86.1% similarity. The type strains of *R. pickettii*, *R. gilardii*, *R. paucula*, *R. solanacearum*, *R. mannitolytica* and *Ralstonia oxalatica* (Şahin *et al.*, 2000) occupied distinct positions. Six isolates grouped together with the type strain of *R. basilensis* in cluster II, above 88.1% similarity. The other metal-resistant isolates were found scattered over clusters IV, V and VI, whereas isolates AB2, AS2, VA1 and VA11 occupied separate positions in the dendrogram. The protein patterns of the latter strains were visually very similar, in apparent contradiction to their locations in the dendrogram. Reproducible protein band distortions were indeed observed above the 116 kDa region for the cluster IV strains and around the 60 kDa region for strains VA10 and VA7 (forming cluster VI), as well as for strains AB2, AS2, VA1 and VA11 (Fig. 2). Little or no band distortion was observed in the protein patterns of the isolates grouping above 85% correlation in cluster V (e.g. the pattern of *R. metallidurans* CH34^T in Fig. 2). The presence of reproducible strain-specific distortions has been described within other species of *Ralstonia* (Vandamme *et al.*, 1999). DNA–DNA hybridization experiments confirmed that these distortions represent intra-species variability and not inter-species variability (see below; Vandamme *et al.*, 1999).

Ralstonia sp. JMP134 and ATCC 17707 occupied separate positions in the dendrogram. Both strains were received originally as *R. eutropha* but, since their whole-cell protein profiles differed clearly from those of the other *R. eutropha* strains (cluster III), we

Table 2. DNA–DNA reassociation values and nucleotide compositions of *R. campinensis*, *R. metallidurans*, *R. basilensis* and *Ralstonia* reference strains

Strain	PAGE cluster	G + C content (mol %)	DNA–DNA reassociation (%) with:*													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. <i>R. campinensis</i> WS2 ^T	I	66.6	100													
2. <i>R. campinensis</i> LH103	I	66.8	100 ^a	100												
3. <i>R. paucula</i> CDC E6793 ^T	–	64.2	31 ^b		100											
4. <i>R. paucula</i> CIP 62.31	–	65†	25 ^a		82†	100										
5. <i>R. basilensis</i> DSM 11853 ^T	II	65.0	18 ^b		17 ^b		100									
6. <i>R. basilensis</i> ER8	II	65.5	14 ^a , 22 ^b		20 ^a , 19 ^b		78 ^b	100								
7. <i>R. basilensis</i> ER121	II	65.4		32 ^b	3 ^a			61 ^a	100							
8. <i>R. metallidurans</i> VA7	VI	ND				29 ^a				100						
9. <i>R. metallidurans</i> CH79	V	63.9		29 ^a	31 ^a	37 ^b				83 ^a	100					
10. <i>R. metallidurans</i> CH34 ^T	V	63.7	26 ^b	37 ^b	35 ^b	39 ^b	16 ^b	17 ^a , 16 ^b	2 ^a , 25 ^b		84 ^a , 88 ^b	100				
11. <i>R. eutropha</i> ATCC 17697 ^T	–	66.6	18 ^a , 26 ^b	41 ^b	21 ^b	24†	20 ^b	22 ^a , 25 ^b	8 ^a , 37 ^b	0 ^a		28 ^b	100			
12. <i>R. solanacearum</i> NCPPB 325 ^T	–	67.0	24 ^a , 11 ^b		16†, 10 ^b	19 ^a	8 ^b	11 ^b	17 ^a		18 ^a	1 ^a , 10 ^b	1 ^a , 13 ^b	100		
13. <i>R. gilardii</i> API 141-02-84 ^T	–	68.3‡		26 ^b	21†				22 ^b			20 ^b	0‡, 40 ^b		100	
14. <i>R. pickettii</i> CCUG 3318 ^T	–	63.8	20 ^a	21 ^a , 18 ^b	13†			20 ^a	5 ^a , 17 ^b			3 ^a , 20 ^b	35 ^a , 25 ^b	32 ^a	21 ^a	100

* Determined by: *a*, initial renaturation rate method (values are means from at least two independent reactions); *b*, microplate method (values are means from at least two reciprocal reactions).

† Data from Vandamme *et al.* (1999).

‡ Data from Coenye *et al.* (1999).

ND, Not determined.

Fig. 3. Neighbour-joining phylogenetic tree of all described *Ralstonia* species, based on 16S rDNA sequence comparisons. Bootstrap values obtained with 500 repetitions are indicated as percentages at all branches. The 16S rDNA sequences of *Alcaligenes faecalis*, *Burkholderia cepacia* and *Pandoraea apista* were included as outgroups.

consider them here as ‘*Ralstonia eutropha*-like’ organisms pending further taxonomic analyses.

DNA–DNA hybridizations and DNA base compositions

Strains representing the protein electrophoretic types were hybridized with type strains of the valid *Ralstonia* species. DNA–DNA reassociation values and nucleotide compositions are shown in Table 2. The type strain of *R. basilensis* showed high DNA reassociation values (61–78 %) with strains ER121 and ER8. These three strains are representatives of protein electrophoretic cluster II and showed only low to intermediate DNA reassociation values (2–37 %) with representatives of other *Ralstonia* species. Hybridization values between *R. campinensis* WS2^T and LH103 (representatives of protein electrophoretic cluster I) were high (100 %), while low to intermediate DNA relatedness (11–41 %) was found between the above-mentioned *R. campinensis* strains and representatives of the other *Ralstonia* species. The metal-resistant strains CH34^T, CH79 and VA7, representing protein electrophoretic clusters V and VI, formed a homogeneous DNA-reassociation group (83–88 %; *R. metallidurans*), showing low to intermediate DNA relatedness (0–39 %) to representatives of other *Ralstonia* species. The high DNA relatedness observed between *R. metallidurans* strains from clusters V and VI confirmed that the protein electrophoretic differences detected reveal intra-species and not inter-species diversity.

The nucleotide compositions presented in Table 2 are in good agreement with those reported for the

Table 3. Comparative fatty acid patterns of *Ralstonia* species

Species are identified as: 1, *R. campinensis* (8 strains); 2, *R. basilensis* (8 strains); 3, *R. metallidurans* (16 strains); 4, *R. eutropha* ATCC 17697^T; 5, *R. solanacearum* NCPPB 325^T; 6, *R. pickettii* CCUG 3318^T; 7, *R. gilardii* API 141-2-84^T; 8, *R. oxalatica* Oxl^T. TR, Fatty acid content below 1%. Summed feature 2 comprises 14:0 3OH, 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 3 comprises 16:1 ω 7c, 15 iso 2OH, 15:0 iso 2OH, 16:1 ω 7c or any combination of these fatty acids.

Fatty acid	1	2	3	4	5	6	7	8
12:0	—	—	—	—	—	TR	TR	—
14:0	5.0 $\pm$ 0.3	4.5 $\pm$ 0.1	4.4 $\pm$ 0.2	2.9	4.2	4.5	4.1	2.6
15:0	—	—	—	TR	TR	TR	TR	TR
15:0 iso	—	—	—	—	—	TR	—	—
16:0	24.6 $\pm$ 1.7	20.6 $\pm$ 0.9	20.9 $\pm$ 1.4	20.6	22.1	25.4	17.7	16.8
16:0 iso	—	—	TR	—	—	—	—	—
17:0	—	—	—	TR	1.5	TR	TR	TR
17:0 cyclo	6.1 $\pm$ 3.5	1.3 $\pm$ 0.9	3.2 $\pm$ 2.1	2.0	2.1	TR	1.2	2.0
18:0	1.5 $\pm$ 4.9	TR	TR	TR	TR	TR	TR	TR
15:1 ω 6c	—	—	—	TR	TR	TR	—	TR
16:1 ω 5c	—	—	—	TR	—	TR	—	TR
18:1 ω 7c	19.5 $\pm$ 1.8	24.7 $\pm$ 1.3	19.8 $\pm$ 2.9	18.5	19.2	20.0	36.4	20.7
19:0 cyclo ω 8c	TR	—	TR	—	—	—	TR	—
14:0 2OH	2.1 $\pm$ 0.2	—	TR	3.2	—	TR	TR	3.8
16:1 2OH	TR	1.8 $\pm$ 0.8	TR	TR	1.3	1.2	—	—
16:0 2OH	TR	TR	3.3 $\pm$ 0.4	—	TR	TR	TR	—
16:0 3OH	—	—	—	TR	—	—	—	—
18:1 2OH	2.7 $\pm$ 0.5	4.8 $\pm$ 0.4	1.4 $\pm$ 0.4	TR	4.0	3.3	2.0	—
Summed feature 2	8.9 $\pm$ 1.4	9.0 $\pm$ 0.5	11.3 $\pm$ 2.5	13.7	9.8	7.7	8.1	15.4
Summed feature 3	28.4 $\pm$ 4.0	32.9 $\pm$ 1.4	34.2 $\pm$ 2.9	36.5	32.7	34.2	27.8	36.6

genus and are not in contradiction with the grouping based on whole-cell protein profiles and DNA–DNA hybridizations.

16S rDNA sequencing

A phylogenetic tree including all *Ralstonia* species is given in Fig. 3. Compared with known representatives of validly described *Ralstonia* species, the 16S rRNA genes of *R. campinensis* WS2^T and *R. metallidurans* CH34^T showed the highest sequence similarity (98.2 and 98.8%) to the 16S rRNA gene of *R. paucula* LMG 3413 (= CIP 62.31; accession no. AF085226), a close relative (82% DNA reassociation) of *R. paucula* CDC E6793^T (Table 2).

The 16S rDNA sequence determined in the present study for *R. basilensis* DSM 11853^T (= LMG 18990^T = LMG 19474^T = RK1^T; accession no. AF312022) differed in 30 base positions (97.9% sequence similarity) from the sequence that was originally deposited for this strain (accession no. AJ002302).

Cellular fatty acids

Table 3 shows the average fatty acid profiles for the *Ralstonia* species examined, which are characterized by large amounts of 16:0 (16.8–25.4%), 18:1 ω 7c

(18.5–36.4%) and summed feature 3 (27.8–36.6%) and moderate amounts of 14:0 (2.6–5.0%) and summed feature 2 (7.7–15.4%).

Discrimination between the different *Ralstonia* species is based mainly on percentages of fatty acids present in trace to very low amounts or completely absent. Furthermore, 18:1 ω 7c seems to be present in *R. gilardii* LMG 5886^T in a significantly higher amount (36.4%) than in all other *Ralstonia* strains examined (13.9–27.9%; raw data not shown).

Phenotypic tests

All strains examined grew at 20, 30 and 37 °C and assimilated D-gluconate, adipate and L-malate; glucose, L-arabinose, D-mannose, D-mannitol and maltose were never assimilated. Indole production from tryptophan and acid formation from glucose were never observed. Enzyme activities detected in all strains examined were alkaline phosphatase, C4 esterase, esterase lipase, leucine arylamidase and acid phosphatase. None of the strains examined showed protease, lipase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase activity. Urease activity and reduction of nitrate were variable. Biochemical features that

Table 4. Biochemical characters that differentiate between *R. campinensis*, *R. basilensis*, *R. metallidurans* and *R. eutropha*

Characters are scored as: +, positive; w, weak; –, negative.

Character	<i>R. campinensis</i>	<i>R. basilensis</i>	<i>R. metallidurans</i>	<i>R. eutropha</i>
Nitrate reduction	+	–*	+*	+†
Nitrite reduction	–	–	+*	–†
Denitrification	–	–	+*	–†
Urease (after 48 h)	+	–	–	–†
Assimilation of citrate	–	+	+	+
Growth at 41 °C	+	–	–	+
Acid phosphatase activity	+*	w	+	+

* One or a few strains showed a different reaction.

† The type strain showed a different reaction.

enable differentiation between the four *Ralstonia* species associated with industrial polluted soils are given in Table 4.

DISCUSSION

A variety of metal-resistant bacteria related to CH34, an *R. eutropha*-like bacterium (Mergeay *et al.*, 1985), were isolated from various industrial metallurgical biotopes (Table 1; Mergeay, 2000). Most of these isolates were examined by means of amplified rDNA restriction analysis (ARDRA) and can be subdivided into two major clusters (Brim *et al.*, 1999). A first cluster contained six isolates obtained from different locations in Belgium, whereas the second cluster contained all other isolates, reference strains of *R. paucula* and several isolates that were received as '*Pseudomonas oxalaticus*'.

R. campinensis sp. nov.

In the present study, the six isolates from ARDRA cluster I (Brim *et al.*, 1999) and two additional ones formed protein electrophoretic cluster I (Fig. 1). Protein electrophoretic cluster I strains were shown to be a homogeneous DNA-hybridization group (Table 2) and could be differentiated further from strains belonging to other *Ralstonia* species by growth at 41 °C, urease activity and citrate assimilation. The DNA G+C content (Table 2) and phylogenetic analysis (Fig. 3) of isolates from cluster I confirmed the allocation of this taxon to the genus *Ralstonia*. We therefore propose the name *R. campinensis* sp. nov. to accommodate these eight isolates.

The 16S rDNA sequence of *R. campinensis* WS2^T (AF312020) is nearly identical (99.9%) to the 16S rDNA sequence of *Ralstonia* sp. CT14, deposited in the EMBL database under accession no. D88001. It is therefore likely that strain CT14 also belongs to the new species *R. campinensis*, although DNA hybridization data are still necessary for a final

assignment at the species level (Stackebrandt & Goebel, 1994).

Differentiation between *R. basilensis*, *R. paucula* and *R. metallidurans* sp. nov.

In the study of Brim *et al.* (1999), the majority of metal-resistant bacteria formed a single ARDRA cluster, including the reference strains of *R. paucula*. In the present study, these isolates were distributed over five protein electrophoretic clusters (II–VI), while the type strain of *R. paucula* occupied a clearly distinct position (Fig. 1). Cluster II was composed of six metal-resistant strains in addition to the type strain of *R. basilensis*, an organism that was described quite recently (Steinle *et al.*, 1998). DNA–DNA hybridizations revealed high DNA–DNA relatedness values between cluster II strains, while low to intermediate values were measured towards reference strains of the other taxa studied (Table 2). Furthermore, strains of cluster II had very similar whole-cell fatty-acid and biochemical profiles. Therefore, we consider all metal-resistant bacteria belonging to protein electrophoretic cluster II as members of *R. basilensis*. The DNA G+C content of the *R. basilensis* strains examined conformed to that of the genus. The 16S rDNA sequence of *R. basilensis* DSM 11853^T determined in the present study and of that of a metal-resistant isolate (ER121) revealed 99.7% sequence identity. However, a reproducible difference of 30 bases was observed between our sequence of the *R. basilensis* type strain and the one deposited in EMBL by Steinle *et al.* (1998). We received a subculture of *R. basilensis* DSM 11853^T twice from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (once as LMG 18990^T and once as LMG 19474^T). Since both subcultures yielded identical whole-cell protein profiles, partial 16S rDNA sequences and repetitive element PCR profiles obtained with the REP and BOX primers (data not shown), we suspect sequencing errors in the sequence deposited by Steinle *et al.* (1998). Alternatively, the

sequence deposited by Steinle may not correspond to the strain that was deposited at the DSMZ. The results of the biochemical analysis seem to favour the last hypothesis, because RK1^T was reported to be arginine dihydrolase- and urease-positive by Steinle *et al.* (1998), while no such enzyme activities were found for DSM 11853^T in our tests.

The 16S rRNA gene of *R. basileensis* DSM 11853^T (accession no. AF312022) showed very high sequence similarities (99.7, 99.9 and 99.6%) to strains *Ralstonia* sp. BKME-6 (accession no. AJ011503), KN1 (accession no. AB031995) and MBIC 3902 (accession no. AB017488). Polyphasic taxonomic analysis and/or DNA–DNA hybridization data are needed in order to elucidate the exact taxonomic relationship between these isolates and *R. basileensis*.

The remaining metal-resistant bacteria that clustered together with *R. basileensis* and *R. paucula* strains in the ARDRA analysis (Brim *et al.*, 1999) formed protein electrophoretic clusters IV, V and VI or occupied distinct positions in the dendrogram (strains AB2, AS2, VA1 and VA11). Visual comparison of their protein profiles (Fig. 2) and DNA–DNA hybridizations (Table 2) showed clearly that these strains represent a new species, for which we propose the name *R. metallidurans* sp. nov. *R. metallidurans* can be differentiated from *R. campinensis*, *R. basileensis* and *R. eutropha* by the presence of the fatty acid 16:0 2OH and from *R. campinensis*, *R. basileensis*, *R. eutropha* and *R. paucula* by the ability to reduce nitrate to nitrogen. *R. metallidurans* CH34^T was included previously in a taxonomy study of *R. eutropha*-like bacteria by Jenni *et al.* (1988). These authors reported a low DNA–DNA hybridization level between strain CH34^T and the *R. eutropha* type strain, which was confirmed in the present study.

Taxonomic status of other *R. eutropha*-like strains

Strains JMP134 (Pemberton *et al.*, 1979) and ATCC 17707 (Davis *et al.*, 1970) were originally classified as *Alcaligenes eutrophus*, now *R. eutropha*. As reported already by Jenni *et al.* (1988), JMP134 shows only low DNA reassociation values (21%) with H20 (= ATCC 17700), a genuine *R. eutropha* strain (cluster III, Fig. 1). This is in good agreement with our results of whole-cell protein profiles (Fig. 1), where the former strain occupied a distinct position in the dendrogram, clearly different from the protein profiles of the *R. eutropha* strains. The same observation holds for ATCC 17707. Therefore, we consider both isolates as *R. eutropha*-like organisms until additional taxonomic data are available.

Description of *Ralstonia campinensis* sp. nov.

Ralstonia campinensis (cam.pin.en'sis. L. adj. *campinensis* of the Kempen or Campine, the geographical region of north-east Belgium where the strains were originally isolated).

Cells are short, motile rods (0.8 × 1.2–1.8 µm), single, in pairs or in short chains, forming round (sometimes with a slightly scalloped margin), smooth, convex and transparent colonies of about 0.5 mm diameter after 24 h of incubation on TSA at 30 °C. Oxidase- and catalase-positive. Grows at 20, 30, 37 and 41 °C, but not at 4 °C. Nitrate is reduced, nitrite is not. No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: urease, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, α-glucosidase (except for strain LH104), protease, β-galactosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Assimilates D-gluconate, caprate, adipate, L-malate and phenylacetate. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl D-glucosamine, maltose or citrate. The DNA G+C content is 66.6–66.8 mol%. Major fatty acid components are 14:0 (5.0%), 16:0 (24.6%), 17:0 cyclo (6.1%), 18:0 (1.5%), 18:1ω7c (19.5%), 14:0 2OH (2.1%), 18:1 2OH (2.7%), summed feature 2 (8.9%) and summed feature 3 (28.4%).

The type strain, WS2^T (= LMG 19282^T = CCUG 44526^T), was isolated from a zinc-desertified area in Lommel, Belgium. Its DNA G+C content is 66.6 mol% and its phenotypic characteristics are as described above for the species. The accession number of its 16S rRNA gene sequence is AF312020.

Description of *Ralstonia metallidurans* sp. nov.

Ralstonia metallidurans (me.tal.li.du'rans. L.n. *metallum* metal; L. pres. part. *durans* enduring; N.L. part. adj. *metallidurans* enduring metal, to indicate that these strains are able to survive high heavy-metal concentrations).

Cells are short, motile rods (0.8 × 1.2–2.2 µm), single, in pairs or in short chains, forming round (sometimes with a slightly scalloped margin), smooth, flat, convex or umbonate and transparent colonies of about 0.5 mm diameter after 24 h of incubation on TSA at 30 °C. Oxidase- and catalase-positive. Grows at 20, 30 and 37 °C, but no growth detected at 4 or 41 °C. An exception is AS2, which does grow at 41 °C. Nitrate and nitrite are reduced (CH34^T reduces nitrate, but not nitrite; VA1 and AS2 reduce neither nitrate nor nitrite). No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, urease (AS2 and AS168 show a weak reaction after 48 h), α-glucosidase, protease, β-galactosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-

acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Assimilation of D-gluconate, caprate (except CH34^T), adipate, L-malate and citrate (except CH34^T). Most strains seem to be able to assimilate phenylacetate after 48 h of incubation [exceptions are AB2 and VA2 (weak assimilation even after 48 h) and CH34^T (no assimilation)]. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl D-glucosamine or maltose. The DNA G+C content is 63.7–63.9 mol%. Major fatty acid components are 14:0 (4.4%), 16:0 (20.9%), 17:0 cyclo (3.2%), 18:1 ω 7c (19.8%), 16:0 2OH (3.3%), 18:1 2OH (1.4%), summed feature 2 (11.3%) and summed feature 3 (34.2%).

The type strain, CH34^T (= LMG 1195^T = DSM 2839^T), was isolated from wastewater from a zinc factory at Liège, Belgium. Its DNA G+C content is 63.7 mol% and its phenotypic characteristics are as described above for the species, unless stated otherwise. The accession number of its 16S rRNA gene sequence is Y10824.

Emended description of *Ralstonia basilensis* Steinle et al. 1998

Cells are short, motile rods (0.8 $\times$ 1.2–2.2 μ m), single, in pairs or in short chains, forming round (sometimes with a slightly scalloped margin), smooth, convex and transparent colonies of about 0.5 mm diameter after 24 h of incubation on TSA at 30 °C. Oxidase- and catalase-positive. Grows at 4, 20, 30 and 37 °C, but no growth detected at 41 °C. No nitrate or nitrite reduction (except strain SV884, which reduces nitrate but not nitrite). No indole production from tryptophan. No glucose fermentation. Enzyme activities detected: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase (weak) and naphthol-AS-BI-phosphohydrolase. Enzyme activities not detected: arginine dihydrolase, urease (ER8 and KS1 give a weak reaction after 48 h), α -glucosidase, protease, β -galactosidase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Assimilation of D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl D-glucosamine or maltose. The DNA G+C content is 65.0–65.5 mol%. Major fatty acid components are 14:0 (4.5%), 16:0 (20.6%), 17:0 cyclo (1.3%), 18:1 ω 7c (24.7%), 16:1 2OH (1.8%), 18:1 2OH (4.8%), summed feature 2 (9.0%) and summed feature 3 (32.9%).

The type strain, RK1^T (= LMG 18990^T = LMG 19474^T = DSM 11853^T), was isolated from a freshwater pond sediment at Amponville, France, after enrichment in a fixed-bed reactor with 2,6-dichlorophenol as the sole carbon and energy source. Its DNA G+C content is 65.0 mol% and its phenotypic characteristics are as described above for the species.

The accession number of the 16S rRNA gene sequence of DSM 11853^T is AF312022.

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