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Revising the taxonomy of the *Acinetobacter lwoffii* group: The description of *Acinetobacter pseudolwoffii* sp. nov. and emended description of *Acinetobacter lwoffii*☆

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ABSTRACT

In 1986, Bouvet and Grimont delineated two related taxa of the genus *Acinetobacter* termed genospecies (GS) 8 and 9. They proposed the name *Acinetobacter lwoffii* for GS8, which included the supposed type strain (CIP 64.10). As the authenticity of CIP 64.10 was later questioned, this study aimed at reassessing the taxonomy of these genospecies. We investigated 52 strains of GS8 or GS9, including CIP 64.10 and the genuine type strain of *A. lwoffii* (NCTC 5866^T). All strains were subjected to the genus-wide comparative analyses of MALDI-TOF whole-cell mass spectra, *rpoB* gene sequences and metabolic traits while whole-genome sequences were analysed for 16 strains. The strains were classified into two distinct groups corresponding to GS8 (n = 15) and GS9 (n = 37). CIP 64.10 fell within GS8 whereas NCTC 5866^T belonged to GS9. Intraspecies ANIb values for the genomes of GS8 (n = 6) and GS9 (n = 10) were ≥96.1% and ≥95.4%, respectively, whereas the ANIb values between them were 86.8–88.6%. Based on core genome phylogeny, GS8 and GS9 formed a distinct clade within the genus, with two respective, strongly supported subclades. GS8 and GS9 were similar in physiological and catabolic properties but were separable by MALDI-TOF MS. We conclude that the name *A. lwoffii* pertains to GS9 and not to GS8 as originally assumed and that these groups represent two species. We propose the name *Acinetobacter pseudolwoffii* sp. nov. for GS8, with ANC 5044^T (= CCM 8638^T = CCUG 67963^T = CIP 111642^T) as the type strain, and provide the emended description of *A. lwoffii*.

Introduction

In their landmark study of 1986 [2], Bouvet and Grimont laid down a basis for the classification of the genus *Acinetobacter* at the species level. Based on DNA-DNA hybridization (DDH) and comprehensive phenotypic testing, they delineated 12 taxa termed genospecies among 85 archive *Acinetobacter* strains. These genospecies were numbered from 1 to 12, with formal species names being proposed for six of them. Two of these genospecies, 8 and 9, were found to be more related to each other than to the other genospecies in terms of both DNA–DNA relatedness and biochemical characteristics. As genospecies 8 included strain CIP 64.10 assumed to be derived from NCTC 5866^T, the type strain of *Acine-*

Abbreviations: MALDI-TOF MS, Matrix-assisted laser desorption/Ionization-time-of-flight mass spectrometry; ANIb, Average nucleotide identity based on BLAST; dDDH, digital DNA–DNA hybridization.

☆ The GenBank/ENA/DDBJ accession numbers for the partial nucleotide sequences of the *rpoB* gene reported in this study are MG564139–MG564159. The whole genome shotgun projects for *Acinetobacter pseudolwoffii* ANC 5044^T, ANC 5318, ANC 5324, and ANC 5347 have been deposited at DDBJ/ENA/GenBank under the accession numbers PHRG00000000, PGPB00000000, PGPA00000000, and PGOZ00000000, respectively. The versions described in this paper are PHRG01000000, PGPB01000000, PGPA01000000, and PGOZ01000000.

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Table 1
Strains of *Acinetobacter lwoffii* (genospecies 9) and *Acinetobacter pseudolwoffii* sp. nov. (genospecies 8).

Strain classification and designation	Specimen[a]	Location and/or year of isolation	Donor and/or reference
Acinetobacter lwoffii (n = 37)			
CCM 5581^{T} = NIPH 512^{T} = CIP 110687^{T} = CNCTC 6167^{T} = NCTC 5866^{T} = Lwoff(1)^{T}	Unknown	Unknown	
NIPH 237	Blood (inpatient)	Příbram, CZ, 1994	[16]
NIPH 238	Vagina (outpatient)	Dobříš, CZ, 1993	[16]
NIPH 393	Throat (outpatient)	Příbram, CZ, 1996	[16]
NIPH 403	Throat (outpatient)	Příbram, CZ, 1996	[16]
NIPH 461	Gastric juices (inpatient)	Praha, CZ, 1996	A. Steinerová [16]
NIPH 473	Nose (outpatient)	Český Brod, CZ, 1996	E. Aldová [16]
NIPH 474	Urine (human)	České Budějovice, CZ, 1996	O. Hausner [16]
NIPH 478 = CIP 110447	Ear (outpatient)	Horní Planá, CZ, 1997	M. Horníková [16]
NIPH 486	Nose (outpatient)	Příbram, CZ, 1997	[16]
NIPH 616	Burn (human)	Praha, CZ, 1994	J. Vránková [16]
NIPH 666	Ear (outpatient)	Praha, CZ, 1997	J. Sobotková [16]
NIPH 671	Cannula (inpatient)	České Budějovice, CZ, 1997	O. Hausner [16]
NIPH 715 = CIP 110448	Pus (inpatient)	Příbram, CZ, 1997	[16]
NIPH 912	Ear (outpatient)	Příbram, CZ, 1998	[16]
NIPH 913	Nose (outpatient)	Sedlčany, CZ, 1998	[16]
NIPH 1094	Nose (outpatient)	Sedlčany, CZ, 1999	[16]
NIPH 2172 = CIP 70.31 = 62[b]	Gangrenous lesion (human)	IT, Before 1946	P. J. M. Bouvet [2]
NIPH 2175 = CIP A162 = 65[b]	Conjunctivitis	Before 1941	P. J. M. Bouvet [2]
NIPH 2176 = CIP 70.19 = 66[b]	Unknown	Unknown	P. J. M. Bouvet [2]
NIPH 2257 = LMG 10590 = 44[b]	Prostate secretion (human)	Malmö, SE, 1980s	I. Tjernberg [31]
NIPH 2266 = LMG 10599 = 202[b]	Urine (human)	Malmö, SE, 1980s	I. Tjernberg [31]
ANC 3906[c]	Mud (forest)	Lány forestland, CZ, 2010	
ANC 4203[c]	Mud (wetland)	Křečkov, CZ, 2011	
ANC 4217[c]	Mud (drained pond)	Hostivice, CZ, 2012	
ANC 4305 = 67[b]	Pus	Unknown	P. J. M. Bouvet [2]
ANC 4309 = 64[b]	Sperm culture	Unknown	P. J. M. Bouvet [2]
ANC 4400 = SH145 = CCUG 57819	Hand (human)	Cologne, DE, 1994	[28]
ANC 4568 = CIP 51.11	Pleural pus (human)	FR, 1951	
ANC 4569 = CIP 102136	Sternum (human)	Paris, FR, 1986	
ANC 4570 = CIP 101966	Sputum (human)	Nevers, FR, 1985	
ANC 4571 = CIP 64.7 = 68[b]	Urine	Before 1964	P. J. M. Bouvet [2]
ANC 4897[c]	Water (forest well)	Bílichov, CZ, 2014	
ANC 5032[c]	Water (river)	Birecik, TR, 2014	
ANC 5055[c]	Water with organic debris (forest creek)	Mohelno, CZ, 2014	
ANC 5085[c]	Soil (dry creekbed)	Peçenek, TR, 2014	
ANC 5086[c]	Soil (dry creekbed)	Peçenek, TR, 2014	
Acinetobacter pseudolwoffii (n = 15)			
ANC 5044^{T}[c] = CCM 8638^{T} = CCUG 67963^{T} = CIP 111642^{T}	Water with organic debris (forest creek)	Mohelno, CZ, 2014	
CIP 64.10 = ANC 4579 = CNCTC 7645	Unknown	Unknown	P. J. M. Bouvet [2]
NIPH 713 = CIP 110446	Vagina (inpatient)	Příbram, CZ, 1997	[16]
NIPH 746 = A46-1[b]	Soil (lakeshore)	Aquilasee, Westfalia, DE, 1990s	H. Seifert
NIPH 748 = A80-2[b]	Water (river)	Centa Albenga, IT, 1990s	H. Seifert
NIPH 831 = RUH 581[b]	Soil	Rotterdam, NL	L. Dijkshoorn [4]
NIPH 1041	Conjunctiva (outpatient)	Příbram, CZ, 1998	[16]
ANC 4683 = CIP 70.17 = 61[b]	Unknown	Before 1958	P. J. M. Bouvet [2]
ANC 5303	Nose (calf)	Rýmařov, CZ, 2015	
ANC 5307	Nose (cow)	Chvalkovice na Hané, CZ, 2015	
ANC 5318	Nose (horse)	Valašské Meziříčí, CZ, 2015	
ANC 5320	Faeces (sheep)	Bělkovice, CZ, 2015	
ANC 5324	Nose (goat)	Kopřivnice, CZ, 2015	
ANC 5347	Rectum (guinea pig)	Ivanovice na Hané, CZ, 2015	
ANC 5504[c]	Mud (wetland)	Olší, CZ, 2016	

Abbreviations: CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection, University of Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; CNCTC, Czech National Collection of Type Cultures, Prague, Czech Republic; LMG, Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK. ANC and NIPH, strain designation used by the Laboratory of Bacterial Genetics. Country abbreviations: CZ, Czech Republic; DE, Germany; FR, France; IT, Italy; NL, the Netherlands; SE, Sweden; TR, Turkey.

[a] If known, hospitalized (inpatient) or ambulatory (outpatient) human patients are indicated.

[b] Strain designation used by the donor.

[c] GPS coordinates of sampling sites: ANC 3906 (50°6′56.602″N, 13°55′11.481″E), ANC 4203 (50°11.11837′N 15°6.88278′E), ANC 4217 (50°4′16.872″N, 14°15′15.190″E), ANC 4897 (50.2506431°N 13.9026414°E), ANC 5032 (37°02′42.0″N 37°58′58.2″E), ANC 5044 and ANC 5055 (49.1043269°N 16.2148017°E), ANC 5085 and ANC 5086 (37°21′50.5″N 41°47′09.5″E), and ANC 5504 (49.1589717°N 15.3853683°E).

tobacter lwoffii [(Audureau 1940 [1]) Brisou and Prévot 1954 [3]], this genospecies was assigned the name *A. lwoffii* in line with Rule 40b of the Bacteriological Code [11].

The authenticity of CIP 64.10 as a derivative of *A. lwoffii* NCTC 5866^{T} was, however, later questioned by Tjernberg and Ursing in their taxonomic study on clinical *Acinetobacter* isolates based on DDH [31]. These authors reported that their results for *A. lwoffii* NCTC 5866^{T} and four strains classified by Bouvet and Grimont [2] as genospecies 8 (ATCC 17925) or 9 (ATCC 17968, ATCC 9957, and ATCC 17910) were discordant with the data of Bouvet & Grimont [2] for CIP 64.10 and the same four ATCC strains. Nevertheless, as the DDH relatedness and ΔT_m values found for all these strains were close to the thresholds recommended for species delineation,

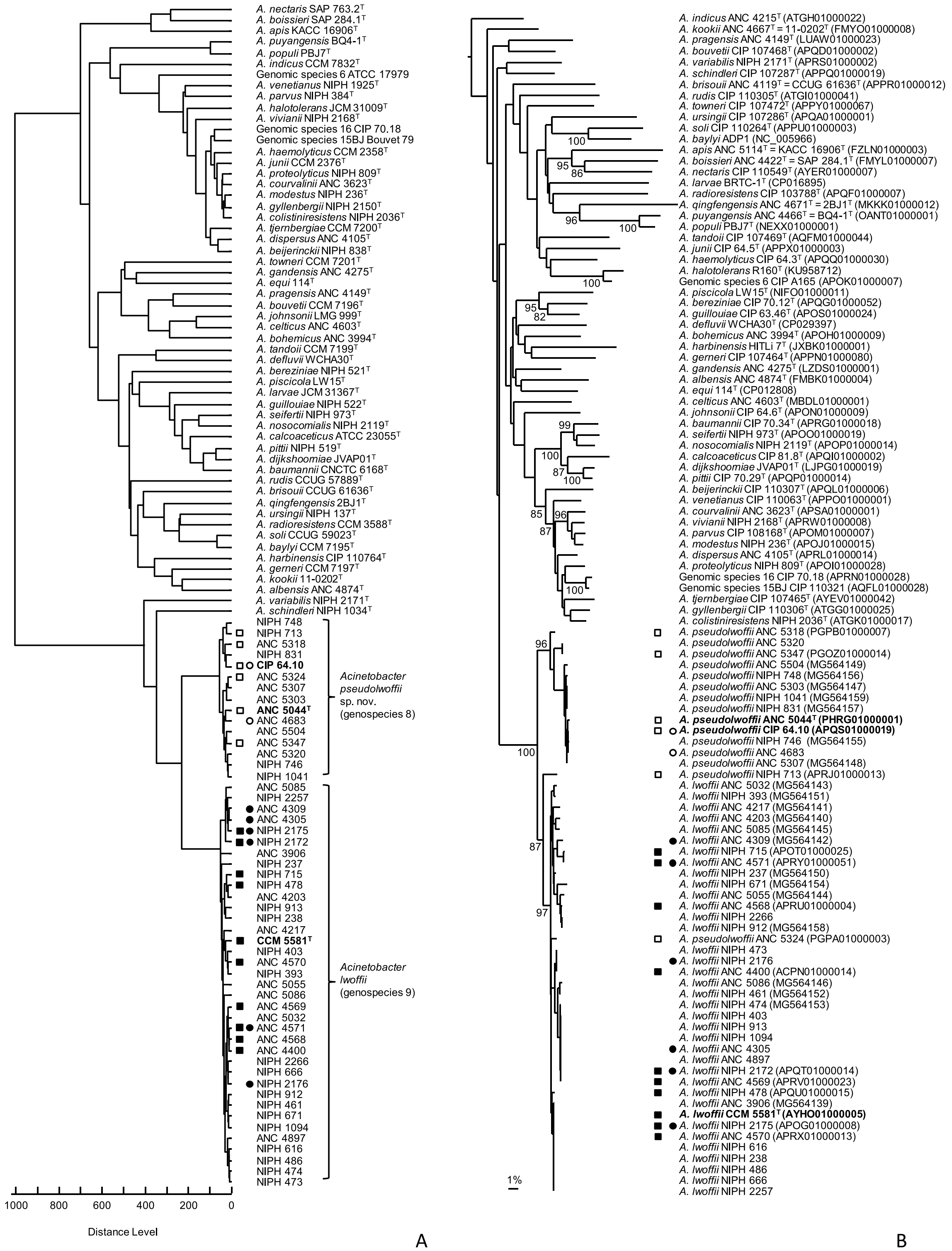

Fig. 1. Results of the clustering of the (A) MALDI-TOF mass spectra and (B) partial sequences of the *rpoB* gene of 37 strains of *Acinetobacter lwoffii* (genospecies 9), 15 strains of *Acinetobacter pseudolwoffii* sp. nov. (genospecies 8) and 56 type or reference strains representing the known species of the genus *Acinetobacter*. MALDI-TOF MS analysis was carried out by using the Biotyper MSP Dendrogram Creation Standard Method (Bruker Daltonics) with the correlation distance measure and average linkage algorithm (UPGMA). Analysis of the *rpoB* sequences was carried out for nucleotide positions 2915–3775 (861 bp) of the coding region of the gene. The tree was reconstructed using the neighbour-joining algorithm with the sequence of *Pseudomonas aeruginosa* PAO1 (DDBJ/ENA/GenBank accession no. NC002516) as the outgroup. GenBank accession numbers for the *rpoB* sequences or whole-genome sequences from which the *rpoB* sequences were extracted are shown in parentheses. In the case of identical *rpoB* sequences, accession numbers are shown only for one representative. Bootstrap values (>80%) after 1000 resamplings are indicated at branch nodes; bar, 1% of change per nucleotide site. Circles and squares denote the strains studied using DNA–DNA hybridization by Bouvet and Grimont [2] and those analysed by whole genome sequencing in the present study, respectively; filled and empty figures indicate the strains allocated to *A. lwoffii* and *A. pseudolwoffii*, respectively.

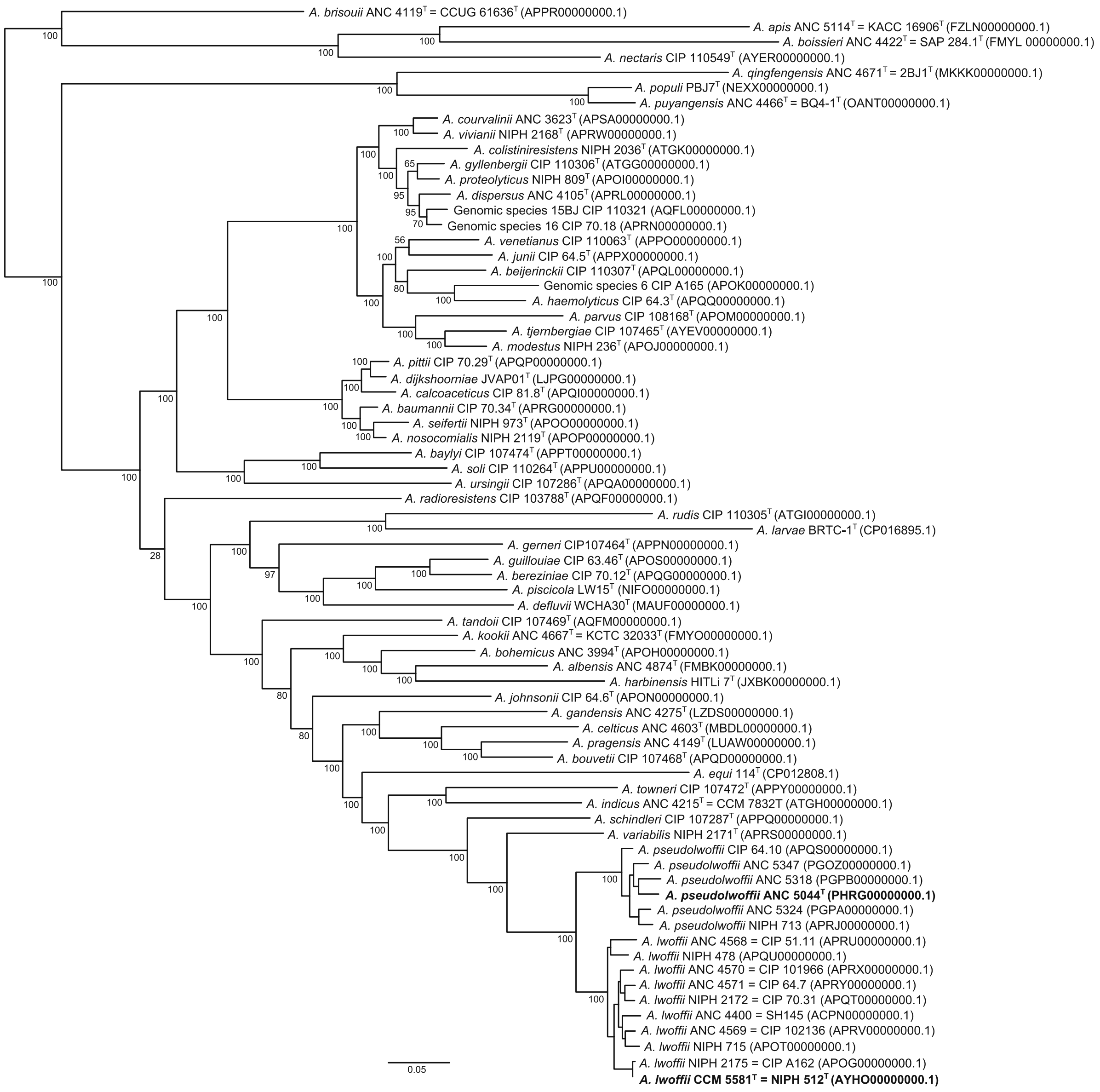

Fig. 2. Core genome-based tree for the genus *Acinetobacter* showing the phylogenetic position of *A. lwoffii* (genospecies 9) and *A. pseudolwoffii* sp. nov. (genospecies 8). Included are the genomes of 10 and 6 strains of *A. lwoffii* and *A. pseudolwoffii*, respectively, and those of 54 type or reference strains representing the known species of the genus *Acinetobacter* (missing is only the genome of *Acinetobacter halotolerans*, not available at the time of analysis). The tree was constructed using maximum likelihood with the PROTGAMMAILGF model for amino acid sequence evolution. Bootstrap values based on 100 replications are shown at the nodes of the tree. Bar, 0.05 amino acid substitutions per site.

they decided to lump them in a single taxonomic group [31]. The fusion of genospecies 8 and 9 into one taxon was followed in many taxonomic studies on *Acinetobacter* [4,6,7,33]. The problem of the identity of CIP 64.10 and *A. lwoffii* NCTC 5866^T was recently reopened by Touchon et al. [32] in their comparative analysis of the whole genome sequences of *Acinetobacter* species. They found that the average nucleotide identity value between the genome sequences of NCTC 5866^T and CIP 64.10 was as low as 88.3%, which indicates that these organisms differed at both the strain and species level of resolution.

Organisms identified as *A. lwoffii* have been commonly reported from human, animal, or environmental specimens [14,15,16,27] and were associated with opportunistic infections in humans [33]. Given this medical and ecological importance, we conducted the present study in order to resolve the taxonomic discrepancies associated with the name *A. lwoffii*.

Material and methods

Bacteria

Fifty-two strains of the *A. lwoffii* group investigated in the present study are listed in Table 1. They included CIP 64.10 and other seven strains studied by Bouvet and Grimont [2], the genuine type strain of *A. lwoffii* (CCM 5581^T derived from NCTC 5866^T) and 43 additional strains which were selected from the collection of the Laboratory of Bacterial Genetics to be as diverse in their origin and phenotypic and genotypic characteristics as possible. All 52 strains had the basic features of the genus *Acinetobacter*: they were oxidase-negative, catalase-positive, and strictly aerobic Gram-stain-negative coccobacilli, incapable of dissimilative denitrification and swimming motility, and positive in the transformation assay of Juni [8]. Representatives of all *Acinetobacter* distinct species with validly published names and several provisional taxa of the genus were included in the following analyses. Three junior synonyms and *Acinetobacter lactucae*, which is synonymous with *Acinetobacter dijkshoorniae*, [19,20,34] were not considered.

MALDI-TOF MS

Whole cell profiling by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed as described previously [24], using a standard matrix based on alpha-cyano-4-hydroxycinnamic acid solution. All measurements and data processing were carried out using the Microflex LT instrument (Bruker Daltonics) and BioTyper software version 3.1 (Bruker Daltonics).

The rpoB *gene analysis*

Comparative nucleotide sequence analysis of the *rpoB* (RNA polymerase β-subunit) gene was carried out for an 861 bp region which corresponds to nucleotide positions 2915–3775 of the *rpoB* coding region of *Acinetobacter baumannii* CIP 70.34^{T} (GenBank/ENA/DDBJ accession no. DQ207471.1) as described previously [17,18]. Cluster analysis was performed using the neighbour-joining algorithm with Kimura's two-parameter substitution model. All analyses were carried out using the BioNumerics 7.6 software (Applied-Maths).

Whole-genome sequence analysis

Generation of DNA sequencing libraries and paired-end sequencing (250 bp × 2) on the Illumina MiSeq platform were carried out in the Genomics Core Facility (EMBL). Reads were assembled *de novo* using the Geneious 9.0.5 software (Biomatters), with only contigs >1000 bp included in the final genome sequences. Genome sequences were compared using the average nucleotide identity based on BLAST (ANIb) and digital DNA–DNA hybridization (dDDH) parameters. ANIb and dDDH values were calculated, respectively, using the JSpecies (http://www.imedea.uib.es/jspecies) [25] and GGDC 2.1 (http://ggdc.dsmz.de) [13] programs with the recommended parameters and/or default settings.

Core genome-based phylogenetic analysis

For phylogenetic analysis based on the *Acinetobacter* core genome, the procedure used by Popel et al. [23] was adopted. All genome sequences were annotated with Prokka [26], and predicted protein coding sequences were used further. To obtain core genes, OrthoMCL [12] was first run to identify orthologs from the genome sequences of five strains, i.e. *Acinetobacter radioresistens* CIP 103788^{T}, *Acinetobacter baylyi* CIP 107474^{T}, *Acinetobacter baumannii* CIP 70.34^{T}, *Acinetobacter calcoaceticus* CIP 81.8^{T}, and *Acinetobacter nosocomialis* NIPH 2119^{T}. Potentially orthologous protein sequences were clustered into homologous groups and redundant sequences were removed to retain only one homologous sequence per genome in each group. Only groups including sequences from all the five strains were analysed further. Sequences within each group were aligned with MAFFT-LINSI [9], and a profile hidden Markov model (pHMM) was created from each alignment using the hmmbuild command of the HMMER3 software package (http://hmmer.janelia.org). HaMStR ortholog search [5] was then applied for the remaining genomes using pHMMs for all homologous groups. Protein sequences found in all genomes were used for phylogeny reconstruction. They were aligned for each group of orthologs with MAFFT-LINSI. Alignments were concatenated, and sites with gaps were trimmed using Phyutility [29]. Maximum Likelihood (ML) tree reconstruction on the resulting

Table 2
Average nucleotide identity based on BLAST (ANIb) and digital DNA–DNA hybridization (dDDH) values for the genome sequences of the *Acinetobacter lwoffii* group.

Genome	Strain	ANIb (%)/dDDH (%)														
		AYHO01	APRU01	APRY01	APQT01	APRX01	APRV01	APOG01	APQU01	APOT01	ACPN01	PHRG01	PGPB01	PGPA01	PGOZ01	APQS01
A. lwoffii (genospecies 9)																
AYHO00000000.1	CCM 5581^{T} (= NIPH 512^{T})															
APRU00000000.1	ANC 4568 (= CIP 51.11)	95.61/65.5														
APRY00000000.1	ANC 4571 (= CIP 64.7)	95.73/66.5	95.4/64.9													
APQT00000000.1	NIPH 2172 (= CIP 70.31)	95.68/66.9	95.69/66.3	96.85/74.2												
APRX00000000.1	ANC 4570 (= CIP 101966)	95.82/67.1	95.58/65.8	96.29/69.9	96.76/72.9											
APRV00000000.1	ANC 4569 (= CIP 102136)	95.77/66.7	95.51/65.4	96.3/70.2	96.68/72.6	96.56/71.6										
APOG00000000.1	NIPH 2175 (= CIP A162)	99.94/99.3	95.6/65.8	95.68/66.9	95.67/67.3	95.79/67.4	95.69/66.8									
APQU00000000.1	NIPH 478	95.64/66.0	95.88/67.2	95.43/64.7	95.76/66.1	95.62/65.3	95.64/66.0	95.61/66.2								
APOT00000000.1	NIPH 715	96.03/68.0	95.69/66.1	96.52/71.9	96.47/70.7	96.49/71.5	96.47/71.0	95.93/67.9	95.77/66.1							
ACPN00000000.1	ANC 4400 (=SH145)	95.86/67.4	95.48/65.1	96.47/71.6	96.63/72.1	96.42/70.8	96.5/71.5	95.83/67.5	95.66/66.1	96.3/69.9						
A. pseudolwoffii (genospecies 8)																
PHRG00000000.1	ANC 5044^{T}	87.32/33.7	87.17/33.1	87.33/34.0	87.42/34.0	87.48/34.1	87.36/33.8	87.28/33.7	87.07/33.0	87.42/34.0	87.3/33.7					
PGPB00000000.1	ANC 5318	87.12/33.3	87.19/33.2	87.29/33.7	87.33/33.7	87.25/33.7	87.18/33.5	87.09/33.3	87.03/32.9	87.25/33.6	87.09/33.5	96.78/73.8				
PGPA00000000.1	ANC 5324	87.34/33.8	87.18/33.2	87.5/34.1	87.4/33.8	87.45/34.0	87.36/33.7	87.28/33.8	87.07/33.1	87.49/33.9	87.31/33.8	97.02/74.8	97.24/77.2			
PGOZ00000000.1	ANC 5347	87.57/34.2	87.16/33.2	87.65/34.6	87.72/34.6	87.69/34.6	87.54/34.2	87.55/34.2	87.34/33.7	87.68/34.6	87.64/34.6	97.12/76.4	97.03/75.9	97.01/75.3		
APQS00000000.1	CIP 64.10	88.33/36.0	88.01/35.1	88.55/36.8	88.55/36.7	88.43/36.6	88.28/36.4	88.21/36.0	87.99/35.3	88.58/36.7	88.46/36.6	96.56/72.0	96.06/68.8	96.45/70.6	96.78/73.5	
APRJ00000000.1	NIPH 713	86.92/33.0	86.92/32.7	87.11/33.3	87.09/33.2	87.06/33.2	87.01/33.0	86.93/33.0	86.76/32.3	87.09/33.2	86.98/33.0	97.41/78.6	97.3/77.0	97.2/76.4	97.47/79.3	96.58/72.6

Table 3
Metabolic and physiological properties of *Acinetobacter lwoffii* (genospecies 9), *Acinetobacter pseudolwoffii* sp. nov. (genospecies 8), and phylogenetically related species.

Characteristic	*A. lwoffii* (n = 37)	*A. pseudolwoffii* (n = 15)	*A. indicus* (n = 2)	*A. schindleri* (n = 22)	*A. towneri* (n = 2)	*A. variabilis* (n = 16)
Growth at 44 °C	–	–	D	–	–	31W (W)
Growth at 41 °C	11W (–)	–	+	+	+	+
Acidification of D-glucose	19 (–)	–	–	–	–	13 (–)
Utilization of						
trans-Aconitate	5 (–)	–	–	–	–	6 (–)
Adipate	76 (+)	40 (+)	–	41 (–)	–	69 (–)
4-Aminobutyrate	84 (+)	–	–	–	–	19 (–)
L-Arabinose	–	–	–	–	–	19 (–)
L-Arginine	5 (–)	–	–	–	–	19 (–)
Azelate	+	80 (+)	50 (–)	64 (–)	–	81 (+)
Benzoate	84 (+)	80 (+)	50 (+)	91 (+)	+	88 (+)
2,3-Butanediol	8 (–)	–	–	32 (–)	50 (–)	81 (+)
Citrate (Simmons)	8 (–)	–	–	59W (+)	–	25 (–)
Ethanol	+	73 (+)	+	95 (+)	+	+
Gentisate	–	–	–	41 (+)	–	–
L-Glutamate	8 (–)	–	50 (+)	- (D)	50 (+)	25 (–)
Glutarate	–	13 (–)	–	95 (+)	–	19 (–)
4-Hydroxybenzoate	–	7 (–)	–	64 (+)	–	–
DL-Lactate	84 (+)	40 (+)	+	+	+	6 (–)
Levulinate	3 (–)	–	–	–	–	–
D-Malate	11 (–)	7 (–)	–	95W (+)	50 (–)	13 (+)
Malonate	8 (–)	7 (–)	–	–	–	–
L-Ornithine	8 (–)	–	–	–	–	–
Phenylacetate	81 (+)	80 (+)	+	–	–	75 (+)
L-Phenylalanine	–	–	–	–	–	38 (–)
D-Ribose	–	–	–	–	–	13 (–)
L-Tartrate	–	13 (+)	–	18 (–)	–	–
Tricarballylate	8 (–)	–	–	45 (+)	–	–

The results were obtained either in this study or have been published previously [10,21]. All strains grew at 20–37 °C and on acetate. None of the strains liquefied gelatin, produced hemolysis on sheep blood agar, or grew on β-alanine, L-aspartate, citraconate, D-gluconate, D-glucose, histamine, L-histidine, L-leucine, putrescine, trigonelline, or tryptamine. *A. lwoffii* strains NIPH 2176, ANC 4305, and ANC 4309 were auxotrophic. +, All strains positive; –, all strains negative; D, (mostly) doubtful or irreproducible reactions; W, (mostly) weakly positive reactions. Numbers are percentages of strains with clearly positive reactions. For strain-dependent reactions, results for type strains are given in parentheses.

supermatrix was then conducted with RAxML 8.1.9 [30] using the PROTGAMMAILGF model for amino acid sequence evolution.

Phenotypic analysis

Metabolic and physiological features were assessed using a genus-targeted set of in-house, strictly standardized tests (Table 3) as described previously [10,18]. Assimilation tests were performed in fluid mineral medium supplemented with 0.1% (w/v) carbon source. In the case of auxotrophic strains, the medium was supplemented with 15% (v/v) of the AUX medium used in the API 20NE system (bioMérieux). Temperature growth tests were carried out in brain-heart infusion broth (Oxoid) using a thermostatically controlled water bath. Except for the temperature growth tests, the culture temperature was 30 °C. The assimilation tests were interpreted after six days of culture and the other tests after three (haemolytic and gelatinase activities) or two (D-glucose acidification, temperature growth tests) days. Gram-staining and tests for oxidase, catalase, nitrate reduction, motility, and anaerobic growth were performed as described by Radolfova-Krizova et al. [24].

Results and discussion

MALDI-TOF MS- and rpoB*-based classification*

The results of the genus-wide cluster analyses of MALDI-TOF whole-cell mass spectra and partial *rpoB* gene sequences are depicted in Fig. 1. Based on MALDI-TOF MS, the 52 strains of the *A. lwoffii* group formed a distinct cluster, with two well separated, internally cohesive subclusters. The larger subcluster (n = 37) included *A. lwoffii* CCM 5581^T and all six strains classified by Bouvet and Grimont as GS9, whereas the smaller one (n = 15) comprised CIP 64.10 and the other strain (CIP 70.17) classified by Bouvet and Grimont as GS8. This picture was mirrored, with the exception of two strains, in the *rpoB*-based phylogram. The partial *rpoB* sequences formed a well separated cluster within the genus, with a large and a small subcluster (respective intracluster sequence identities of ≥96.9% and ≥97.3%), which comprised, respectively, the strains classified by Bouvet and Grimont as GS9 and GS8. The two strains with inconsistent positions in the *rpoB* and MALDI-TOF MS dendrograms were NIPH 713, placed in between two main clusters in the *rpoB* tree, and ANC 5324, which grouped with the GS8 and GS9 strains in the MALDI-TOF MS and *rpoB* dendrograms, respectively. However, based on the analysis of whole genome sequences (Table 2, Fig. 2), these two strains could be allocated unequivocally to the GS8 group, and the same picture was obtained if the complete *rpoB* sequences derived from the whole genomes were compared. The inspection of the completete *rpoB* sequences of NIPH 713 and ANC 5324 revealed that while the major parts of these sequences were congruent with those of the GS8 strains, the regions used for the partial *rpoB* gene analysis (positions 2915–3775 of the *rpoB* coding region) were more similar, either completely (ANC 5324) or partly (NIPH 713), to those of the GS9 strains. This suggests that the *rpoB* sequences of these two strains underwent homologous recombination following the acquisition of the *rpoB* sequences from strains of the GS9 group.

Comparison of whole genome sequences

Whole genome sequences were analysed in six and 10 strains classified, respectively, as GS8 and GS9 by Bouvet and Grimont and/or by MALDI-TOF MS and *rpoB* sequencing (Table 2). Four of them (GenBank/ENA/DDBJ accession nos PGPA00000000.1, PGPB00000000.1, PGOZ00000000.1, and PHRG00000000.1) were determined in the present study, whereas the remaining 12 were published previously [22,32]. The basic features of these genome sequences are summarized in Table S1. The pairwise

Table 4
Description of *Acinetobacter pseudolwoffii* sp. nov. and emended description of *Acinetobacter lwoffii* according to Digital Protologue TA00592 and TA000713, respectively, assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00592	TA00713
Type of description	New description	Taxon emendation
Former taxonumbers of the protologues subjected to emendation		TA00593
Date of valid publication		1980-01-01
Validation list		Approved lists of bacterial names
IJSEM volume & pages		30: 225–420
Species name	*Acinetobacter pseudolwoffii*	*Acinetobacter lwoffii*
Genus name	*Acinetobacter*	*Acinetobacter*
Specific epithet	*pseudolwoffii*	*lwoffii*
Species status	sp. nov.	sp. nov.
Species etymology	pseu.do.lwoff'i.i. Gr. adj. pseudes false; N.L. gen. masc. n. *lwoffii*, of Lwoff, named in honour of André Lwoff and specific epithet; N.L. masc. adj. *pseudolwoffii*, a false (*Acinetobacter*) *lwoffii*, referring to the high phenotypic similarity to and historical confusion with *A. lwoffii*	lwoff'i.i.; N.L. gen. masc. n. *lwoffii*, of Lwoff, named in honour of André Lwoff
Authors	Alexandr Nemec, Lenka Radolfová-Křížová, Martina Maixnerová, Matěj Nemec, Dominique Clermont, Jaroslav Bzdil, Petr Ježek, Petra Španělová	Alexandr Nemec, Lenka Radolfová-Křížová, Martina Maixnerová, Matěj Nemec, Dominique Clermont, Jaroslav Bzdil, Petr Ježek, Petra Španělová
Title	Revising the taxonomy of the *Acinetobacter lwoffii* group: the description of *Acinetobacter pseudolwoffii* sp. nov. and emended description of *Acinetobacter lwoffii*	Revising the taxonomy of the *Acinetobacter lwoffii* group: the description of *Acinetobacter pseudolwoffii* sp. nov. and emended description of *Acinetobacter lwoffii*
Journal	Systematic and Applied Microbiology	Systematic and Applied Microbiology
Volume & pages	Submitted	Submitted
Corresponding author	Alexandr Nemec	Alexandr Nemec
E-mail of the corresponding author	alexandr.nemec@szu.cz	alexandr.nemec@szu.cz
Submitter	Alexandr Nemec	Alexandr Nemec
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Designation of the type strain	ANC 5044	NCTC 5866
Strain collection numbers	CCM 8638 = CIP 111642 = CCUG 67963 = CNCTC 7472	CCM 5581 = CIP 110687 = CNCTC 6167 = NIPH 512
16S rRNA gene accession number	PHRG01000001.1	AYHO01000004.1
Genome accession number [EMBL]	PHRG00000000.1	AYHO00000000.1
Genome status	Draft	Draft
Genome size	3105.311	3382.003
GC mol%	43.3	43.1
Country of origin	Czech Republic	Unknown
Region of origin	Moravia	Unknown
Source of isolation	Creek sediment from a deciduous forest	Unknown
Sampling date	2014-09-28	1939-01-01
Geographic location	Natural reserve Mohelnička	
Latitude	49°6′15.577″N	
Longitude	16°12′53.286″E	
Number of strains in study	15	37
Source of isolation of non-type strains	Forest water with organic debris, river water, soil, wetland mud, human (conjuctiva, vagina), calf (nose), cow (nose), horse (nose), sheep (faeces), goat (nose), guinea pig (rectum)	Human (blood, burn, cannula, conjunctiva, ear, gangrenous lesion, gastric juices, hand, nose, prostate secretion, pus, sperm, sputum, sternum, throat, urine, vagina), forest or wetland mud, forest or river water, soil
Growth medium, incubation conditions [temperature, pH, and further information] used for standard cultivation	Tryptic soy agar, nutrient agar or other ordinary agar media; culturable at ≈30 °C and pH of ≈7.0	Tryptic soy agar, nutrient agar or other ordinary agar media; culturable at ≈30 °C and pH of ≈7.0
Is a defined medium available	Int. J. Syst. Evol. Microbiol. 2009; 59, 118–124	Int. J. Syst. Evol. Microbiol. 2009; 59, 118–124
Conditions of preservation	Lyophilisation or storage in broth with glycerol (50% by volume) at temperatures from −80 °C to −10 °C	Lyophilisation or storage in broth with glycerol (50% by volume) at temperatures from −80 °C to −10 °C
Gram stain	Negative	Negative
Motility	Nonmotile	Nonmotile
Colony morphology	Colonies on tryptic soy agar (Oxoid) after incubation at 30 °C for 24 h are 1.0-2.0 mm in diameter, grey-white, slightly opaque, circular, convex and smooth, with entire margins; neither haemolysis nor greenish discoloration is observed on agar media supplemented with sheep erythrocytes	Colonies on tryptic soy agar (Oxoid) after incubation at 30 °C for 24 h are 1.0-2.0 mm in diameter, grey-white, slightly opaque, circular, convex and smooth, with entire margins; neither haemolysis nor greenish discoloration is observed on agar media supplemented with sheep erythrocytes
Temperature range	15–37	15–37
pH category	Neutrophile	Neutrophile
Relationship to O_2	Aerobe	Aerobe
O_2 conditions for strain testing	Aerobiosis	Aerobiosis
Carbon source used [specific compounds]	Acetate	Acetate, azelate, ethanol
Carbon source not used [specific compounds]	*trans*-Aconitate, 4-aminobutyrate, β-alanine, L-arabinose, L-arginine, L-aspartate, 2,3-butanediol, citraconate, citrate (Simmons), gentisate, D-gluconate, D-glucose, L-glutamate, histamine, L-histidine, L-leucine, levulinate, L-ornithine, L-phenylalanine, putrescine, D-ribose, tricarballylate, trigonelline, tryptamine	β-Alanine, L-arabinose, L-aspartate, citraconate, gentisate, D-gluconate, D-glucose, glutarate, histamine, L-histidine, 4-hydroxybenzoate, L-leucine, L-phenylalanine, putrescine, D-ribose, L-tartrate, trigonelline, tryptamine

Table 4 (*Continued*)

Taxonumber	TA00592	TA00713
Carbon source variable [specific compounds]	Adipate, azelate, benzoate, ethanol, glutarate, 4-hydroxybenzoate, DL-lactate, D-malate, malonate, phenylacetate, L-tartrate	*trans*-Aconitate, adipate, 4-aminobutyrate, L-arginine, benzoate, 2,3-butanediol, citrate (Simmons), L-glutamate, DL-lactate, levulinate, D-malate, malonate, L-ornithine, phenylacetate, tricarballylate
Acid formation from carbohydrates (variable)		D-Glucose (oxidative)
Acid formation for carbohydrates (all negative)	D-Glucose	
Nitrogen source	Amonium	Amonium
Terminal electron acceptor	Oxygen	Oxygen
Energy metabolism	Chemoorganotroph	Chemoorganotroph
Oxidase	Negative	Negative
Catalase	Positive	Positive
Negative tests	Gelatin hydrolysis	Gelatin hydrolysis
Habitat	Ubiquitous	Ubiquitous
Biotic relationship	Free-living	Free-living
Known pathogenicity		Opportunistic
MIiscellaneous, extraordinary features relevant for the description	Positive in a transformation assay [J. Bacteriol. 1972; 112, 917-931]; the genome sequence (PHRG00000000.1) of the type strain contains one complete copy of the 16S rRNA gene (PHRG01000001.1, locus_tag: CWI32_02650)	Positive in a transformation assay [J. Bacteriol. 1972; 112, 917-931]; the genome sequence (AYHO00000000.1) of the type strain contains five copies of the 16S rRNA gene, with three sequence variants, i.e. AYHO01000004 (locus_tag: P800_01948), AYHO01000005 (locus_tag: P800_02544 and P800_02592)

ANIb and dDDH values for the 16 genome sequences are shown in Table 2 while those between these strains and all hitherto described *Acinetobacter* species are summarized in Tables S2 and S3. The intraspecies ANIb/dDDH values for GS8 and GS9 were 96.06–97.47%/68.8–79.3% and 95.4–99.94%/64.7–99.3%, respectively, whereas the ANIb/dDDH values between GS8 and GS9 were 86.76–88.58%/32.3–36.8%. The ANIb and dDDH values between the genomes of GS8 or GS9 and those of the other species of the genus were ≤82.84% and ≤26.9%, respectively. In light of the recommended threshold values of ANIb (95–96%, [25]) and dDDH (70% [13]) for species circumscription, these values indicate that GS8 and GS9 are two distinct species. Although a high proportion of the pairwise dDDH values for GS9 were slightly below 70% (Table 2), it is of note that such dDDH values can also be found for the members of other ecologically ubiquitous species such as *Acinetobacter johnsonii* (unpublished results).

Core genome-based phylogeny

The results of the phylogenetic analysis of the 16 genome sequences of the *A. lwoffii* group within the whole genus are shown in Fig. 2. The resulting phylogram was constructed from the concatenated alignment of the of 372,478 amino acid residues based on 1266 orthologous protein groups. The 16 genomes formed a distinct clade within the genus, with two monophyletic subbranches which, respectively, correspond to GS8 and GS9. This picture is consistent with the previously published phylogram [32] based on 1590 protein families of the *Acinetobacter* core genome, which included 11 of the 16 genome sequences of the *A. lwoffii* group used in the present study.

Physiological and metabolic features

Phenotypic features assessed using the genus-targeted set of physiological and metabolic tests (Table 3) are presented in the standardized way as described in our previous nomenclatural proposals [10,21]. Table S4 shows the summarized data for GS8 and GS9, along with those for all known *Acinetobacter* species with validly published names, whereas Table 3 is a subset of these data, which compares the phenotypes of GS8 and GS9 with those of the species that are phylogenetically closest to them (*Acinetobacter indicus*, *Acinetobacter schindleri*, *Acinetobacter towneri*, and *Acinetobacter variabilis*) according to the phylogram of Fig. 2. Overall, GS8 and GS9 belong, together with those phylogenetically close species, to catabolically less active members of the genus, with a limited number of characteristics that can differentiate between them. Even though no single diagnostic feature was identified which could discriminate unequivocally between the strains of GS8 and GS9, GS9 appeared to be more active than GS8 as exemplified by a higher proportion of GS9 strains to grow on adipate, 4-aminobutyrate, DL-lactate, and ethanol or by the ability of some GS9 strains to oxidize D-glucose (Table 3).

The type strain of A. lwoffii

To our best knowledge, the National Collection of Type Cultures preserves the oldest known lineage of the type strain of *A. lwoffii* (listed under no. NCTC 5866^{T}), deposited there by André Lwoff before 1939. NCTC 5866^{T} was later deposited in a number of culture collections including the Collection de l'Institut Pasteur (CIP 64.10) and the Czech Collection of Microorganisms (CCM 5581^{T}). However, the results of the present study showed that CIP 64.10 and CCM 5581^{T} differed at both the strain and species level. To shed more light on, we obtained another culture of NCTC 5866^{T} from the National Collection of Type Cultures, which has been deposited in the Collection de l'Institut Pasteur under no. CIP 110687^{T}. This new culture was studied along with CIP 64.10 and CCM 5581^{T} using macrorestriction analysis with ApaI, which corroborated the identity of CIP 110687^{T} and CCM 5581^{T} and distinctness of CIP 64.10 (data not shown). These observations are in line with those of Tjernberg and Ursing [31] and indicate that CIP 64.10 is a strain different from NCTC 5866^{T}. Although a mislabelling of strains is the most likely reason behind this, it must be emphasized that such a mislabelling was difficult to recognize at the time of the study of Bouvet and Grimont [2], given the high phenotypic similarity of the two strains (Table 3). To avoid further confusion, CIP 64.10 is not available from the Collection de l'Institut Pasteur any longer but has been deposited in the Czech National Collection of Type Cultures under no. CNCTC 7645.

Conclusions

The present study provides strong evidence that taxa GS8 and GS9 described by Bouvet and Grimont [2] are two phylogenetically related but taxonomically distinct species of the genus *Acinetobacter*. This evidence is based on the congruence of genotypic and phenotypic results, with those based on the analyses of whole-genome sequences and whole-cell protein spectra being most conclusive. The two species are ecologically ubiquitous, as indicated by their occurrence in various human, animal, and environmental specimens. Despite their mutual resemblance in catabolic properties and possible problems with their identification based on single gene markers, the reliable differentiation between these species for routine diagnostics is achievable via MALDI-TOF MS. Our results further demonstrate that the genuine type strain of *A. lwoffii* [1,3] is a member of GS9 and not GS8, as assumed by Bouvet and Grimont [2] and that, therefore, the name *A. lwoffii* pertains to the former genospecies. In light of these findings, we provide the emended description of *A. lwoffii* and formal proposal of the name *Acinetobacter pseudolwoffii* sp. nov. for GS8, which are given in Table 4 with respective Taxonumbers TA00713 and TA00592.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.10.004.

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