Acinetobacter rudis sp. nov., isolated from raw milk and raw wastewater

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Two bacterial strains, G30^T and A1PC16, isolated respectively from raw milk and raw wastewater, were characterized using a polyphasic approach. Chemotaxonomic characterization supported the inclusion of these strains in the genus *Acinetobacter*, with Q-8 and Q-9 as the major respiratory quinones, genomic DNA G+C contents within the range observed for this genus (38–47 mol%) and $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH as the predominant fatty acids. The observation of 16S rRNA gene sequence similarity lower than 97 % with other *Acinetobacter* species with validly published names led to the hypothesis that these isolates could represent a novel species. This hypothesis was supported by comparative analysis of partial sequences of the genes *rpoB* and *gyrB*, which showed that strains G30^T and A1PC16 did not cluster with any species with validly published names, forming a distinct lineage. DNA–DNA hybridizations confirmed that the two strains were members of the same species, which could be distinguished from their congeners by several phenotypic characteristics. On the basis of these arguments, it is proposed that strains G30^T and A1PC16 represent a novel species, for which the name *Acinetobacter rudis* sp. nov. is proposed. The type strain is strain G30^T (=LMG 26107^T =CCUG 57889^T =DSM 24031^T =CECT 7818^T).

Reflecting their ubiquity and metabolic versatility, bacteria of the genus *Acinetobacter* have been isolated from environmental sources as diverse as, for example, human and animal clinical samples, activated sludge and seawater (Carr *et al.*, 2003; Juni, 2005; Nemec *et al.*, 2009; Vaneechoutte *et al.*, 2009). Some *Acinetobacter* species are also psychrotolerant, as demonstrated by their occurrence in low-temperature habitats such as frozen vegetables and fish products (Gennari & Stegagno, 1985, 1986) or refrigerated raw milk (Uraz & Çitak, 1998; Munsch-Alatossava & Alatossava, 2006; Hantsis-Zacharov & Halpern, 2007). At the time of writing, the diversity of this genus supported the definition of 22 validly published species names (Euzéby, 1997). The current work characterized two bacterial strains of the genus *Acinetobacter*, G30^T and A1PC16, which were respectively isolated from raw milk and raw wastewater in independent studies in locations separated by more than 4000 km (Hantsis-Zacharov & Halpern, 2007; Novo & Manaia, 2010). This curious observation, allied with the fact that neither of the isolates could be identified as a member of any *Acinetobacter* species with a validly published name, motivated the present study, which aimed at comparing the two organisms and characterizing a presumed novel species.

Strain G30^T was isolated in northern Israel from raw milk, on plate count agar (PCA; Pronadisa), during a study of the seasonal dynamics of culturable psychrotolerant communities in that product (Hantsis-Zacharov & Halpern, 2007). Strain A1PC16 was isolated in northern Portugal from raw wastewater on PCA supplemented with 4 mg ciprofloxacin

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *gyrB* gene sequences of strains G30^T and A1PC16 are respectively EF204258 and FN298236 (16S rRNA gene), FN393751 and FN393750 (*rpoB*) and FN597721 and FN597720 (*gyrB*).

Two supplementary tables are available with the online version of this paper.

l^{-1} during a study on antibiotic resistance prevalence in municipal wastewater (Novo & Manaia, 2010). Both strains were purified by subculturing and maintained on PCA. Cultures were incubated at 30 °C and cryopreserved at −80 °C in nutrient broth supplemented with 15 % (v/v) glycerol.

Although limitations of the 16S rRNA gene sequence to discriminate species are recognized within some genera, analysis of this gene is still a method of choice to infer relationships at the genus level, and this was our first approach. The following type strains that presented more than 95 % 16S rRNA gene sequence similarity with the isolates under study were included in the phenotypic comparison: *Acinetobacter baumannii* CCUG 19096^T, *A. baylyi* CCUG 50765^T, *A. bereziniae* LMG 1003^T, *A. calcoaceticus* CCUG 12804^T, *A. guillouiae* LMG 988^T, *A. johnsonii* CCUG 19095^T, *A. junii* CCUG 889^T, *A. radioresistens* CCUG 56440^T and *A. venetianus* CCUG 45561^T.

Colony and cell morphology, the Gram-stain reaction, cytochrome *c* oxidase and catalase activities and motility were analysed based on the methodologies of Murray *et al.* (1994) and Smibert & Krieg (1994). Unless otherwise stated, all biochemical and physiological tests were performed as described by Vaz-Moreira *et al.* (2007). Phenotypic profiling was performed, using the API 20E, API 20NE, API 32GN and API ZYM panels (bioMérieux), following the instructions of the manufacturer, and the results were observed after 48 h. To confirm the results obtained for the assimilation of sole carbon sources, assimilation of D-glucose, L-proline, L-alanine, L-histidine, L-arabinose, lactic acid, propionic acid, sodium citrate and valeric acid was also tested in the basal mineral medium of Cruze *et al.* (1979) supplemented with 0.1 % (w/v) of each carbon source as described by Nemec *et al.* (2009). Additionally, the carbon sources L-arginine, putrescine, ethanol, 2,3-butanediol, L-aspartic acid, L-leucine, L-phenylalanine, sodium benzoate and histamine, which are not available in the commercial panels referred to above, were tested under these conditions. Simmons' citrate was tested on Simmons' citrate agar (Pronadisa), incubated for 48 h at 30 °C. Aerobic acid production from glucose was tested with the Hugh & Leifson OF basal medium (Pronadisa). Haemolytic activity was tested on Columbia agar with 5 % sheep blood (bioMérieux).

For cellular fatty acid analysis, strains were cultured on tryptic soy agar (Difco) for 24 h at 28 °C and the fatty acids were extracted and methylated as described by Ben-Ze'ev *et al.* (2005). Fatty acid methyl esters were analysed by gas chromatography using the MIDI/Hewlett Packard Microbial Identification System (Analytical Services Inc.). The G+C content of genomic DNA and the respiratory quinones were analysed as described previously (Vaz-Moreira *et al.*, 2007), using the methods of Mesbah *et al.* (1989) and Tindall (1989), respectively.

Genomic DNA was isolated according to the method of Marmur (1961). DNA–DNA hybridizations were performed, in duplicate, using a non-radioactive method, as described by Ziemke *et al.* (1998). DNAs of strain G30^T and A1PC16 were double-labelled with DIG-11–dUTP and biotin-16–dUTP, using a nick translation kit (Boehringer Mannheim). Each labelled DNA was hybridized against itself and, as a control, against DNA from *A. baylyi* CCUG 50765^T.

The nucleotide sequence of the nearly complete 16S rRNA gene (1500 and 1489 bp for strains G30^T and A1PC16) was determined after PCR amplification from genomic DNA extracts, as described previously (Ferreira da Silva *et al.*, 2007; Hantsis-Zacharov & Halpern, 2007). The region Z1–Z2 of the *rpoB* gene was examined as described before (La Scola *et al.*, 2006). The *gyrB* gene was amplified with primers APRU-F/UP1E-R and sequenced with primers M13R/M13(−21) (Yamamoto *et al.*, 1999), in the following reaction mixture: 2 U *Taq* polymerase (Fermentas), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.8 μM each primer and 2 μl crude cell lysate, in a total volume of 50 μl. After 2 min at 94 °C, samples were subjected to 30 cycles of amplification, 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and a final extension step of 2 min at 72 °C. The nucleotide sequences of the 16S rRNA, *rpoB* and *gyrB* genes were compared with reference sequences available in the GenBank/EMBL/DDBJ database using BLAST from NCBI (http://www.ncbi.nlm.nih.gov). Sequence analyses were conducted using the software MEGA 4.0 (Tamura *et al.*, 2007). Sequence similarities were estimated based on the model of Jukes & Cantor (1969) and dendrograms of estimated phylogenetic relationships were created using the neighbour-joining and maximum-parsimony methods. For dendrogram construction, non-homologous and ambiguous nucleotide positions were excluded from the calculations.

16S rRNA gene sequence analysis showed the affiliation of strains G30^T and A1PC16 to the genus *Acinetobacter*. This result was supported by the chemotaxonomic characterization. As described for the genus *Acinetobacter* (Collins & Jones, 1981), strains G30^T and A1PC16 contained ubiquinones Q-8 and Q-9 as the major respiratory quinones, respectively representing 14 and 86 % in strain G30^T and 12 and 88 % in strain A1PC16. The G+C content of the genomic DNA of strains G30^T and A1PC16 was within the range observed for this genus (38–47 mol%) (Juni, 2005), with values of 39.3 ± 0.05 and 39.7 ± 0.12 mol%, respectively. In the cellular fatty acid compositions of strains G30^T and A1PC16, as well as those of the type strains of related species, the components $C_{18:1}\omega9c$, summed feature 3 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{12:0}$ predominated (Supplementary Table S1, available in IJSEM Online). The profile observed is consistent with the cellular fatty acid composition reported by Kämpfer (1993), particularly in the presence of $C_{12:0}$ 2-OH, supporting the inclusion of strains G30^T and A1PC16 in the genus *Acinetobacter*.

Strains G30^T and A1PC16 shared 100 % 16S rRNA gene sequence similarity, suggesting that the two strains could be members of the same species. In fact, DNA–DNA hybridization between the two strains resulted in a value of

82.4 % (reciprocal 95.6 %), above the threshold of 70 % established by Wayne *et al.* (1987).

Although related to members of the genus *Acinetobacter*, strains G30^T and A1PC16 showed less than 97 % 16S rRNA gene sequence similarity to the type strains of all species of this genus with validly published names. Based on 16S rRNA gene sequence similarity, the closest neighbours of strains G30^T and A1PC16 included the type strains of *A. venetianus* (96.5 %), *A. guillouiae* (96.4 %), *A. bereziniae* (96.3 %), *A. radioresistens* (96.0 %), *A. junii* (95.8 %), *A. baumannii* (95.8 %), *A. calcoaceticus* (95.5 %), *A. johnsonii* (95.4 %) and *A. baylyi* (95.2 %). The highest 16S rRNA gene sequence similarities were below 97 %, confirming our initial hypothesis that the isolates under study could not be affiliated to any species of the genus *Acinetobacter* with a validly published name (Fig. 1). It has been extensively argued and demonstrated that organisms sharing less than 97 % 16S rRNA gene sequence similarity will yield DNA–DNA hybridization values lower than 60 % (Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996; Stackebrandt & Ebers, 2006) and thus cannot be considered members of the same species. Hence, DNA–DNA hybridizations with other members of the genus were not necessary in this case. The relationship of strains G30^T and A1PC16 with species with validly published names and genomic species of the genus *Acinetobacter* was also assessed on the basis of the comparison of partial *rpoB* and *gyrB* gene sequences; the usefulness of these sequences in species definition in the genus *Acinetobacter* has been demonstrated previously (Yamamoto *et al.*, 1999; La Scola *et al.*, 2006). The constructed dendrograms included the type strains of the species of the genus and representatives of different genomic species (Figs 2 and 3). Both of these analyses demonstrated that the proposed novel species forms a distinct lineage within the genus *Acinetobacter*. Strains G30^T and A1PC16 shared less than 85 and 82 % sequence similarity, respectively, for the genes *rpoB* and *gyrB*, with the type strains of species with validly published names and strains of the different genomic species. In contrast, identity of 99.8 and 99.3 %, respectively, for the partial *rpoB* and *gyrB* gene sequences was observed between strains G30^T and A1PC16. These analyses confirmed the results of 16S rRNA gene sequence comparison, consistently showing that strains G30^T and A1PC16 form a distinct clade within the genus *Acinetobacter* (Figs 1, 2 and 3).

Strains G30^T and A1PC16 and the type strains of closely related species were characterized in parallel in the current study (Table 1). Phenotypically, the non-haemolytic strains G30^T and A1PC16 were Gram-negative coccobacilli that were strictly aerobic, catalase-positive and oxidase-negative and non-motile, characteristic of the genus *Acinetobacter* (Juni, 2005). Despite being isolated from different habitats and in different regions, strains G30^T and A1PC16 responded similarly to all the phenotypic tests performed. These strains could be distinguished from the other type strains examined in this study by the inability to assimilate L-proline. Other distinctive traits determined in this study included the following: from *A. venetianus* CCUG 45561^T, assimilation of L-arginine, 2,3-butanediol, phenylacetate, L-phenylalanine, lactic acid, 3-hydroxybutyric acid and 4-hydroxybenzoic acid

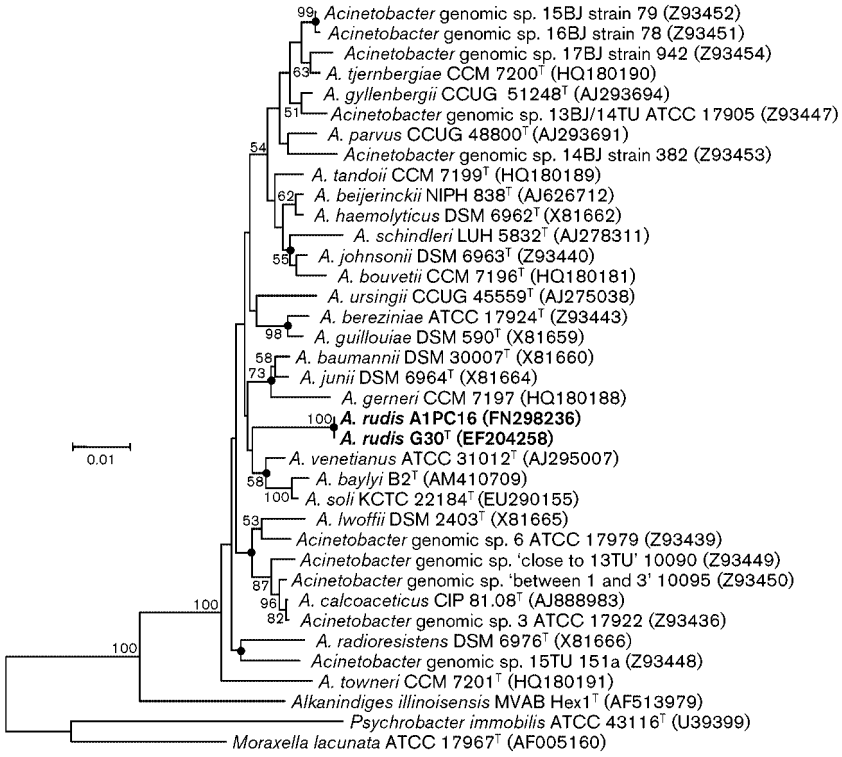

Fig. 1. Rooted tree based on 16S rRNA gene sequences, showing the nearest neighbours of strains G30^T and A1PC16. The region used corresponds to positions 100–1423 (*Escherichia coli* numbering). The tree was generated by the neighbour-joining method. The sequences of *Psychrobacter immobilis* ATCC 43116^T and *Moraxella lacunata* ATCC 17967^T were used as an outgroup. Bootstrap percentages, generated from 1000 resamplings, ⩾50 % are indicated at branch points. Filled circles indicate branches also recovered by the maximum-parsimony method. Bar, 1 substitution per 100 nucleotide positions.

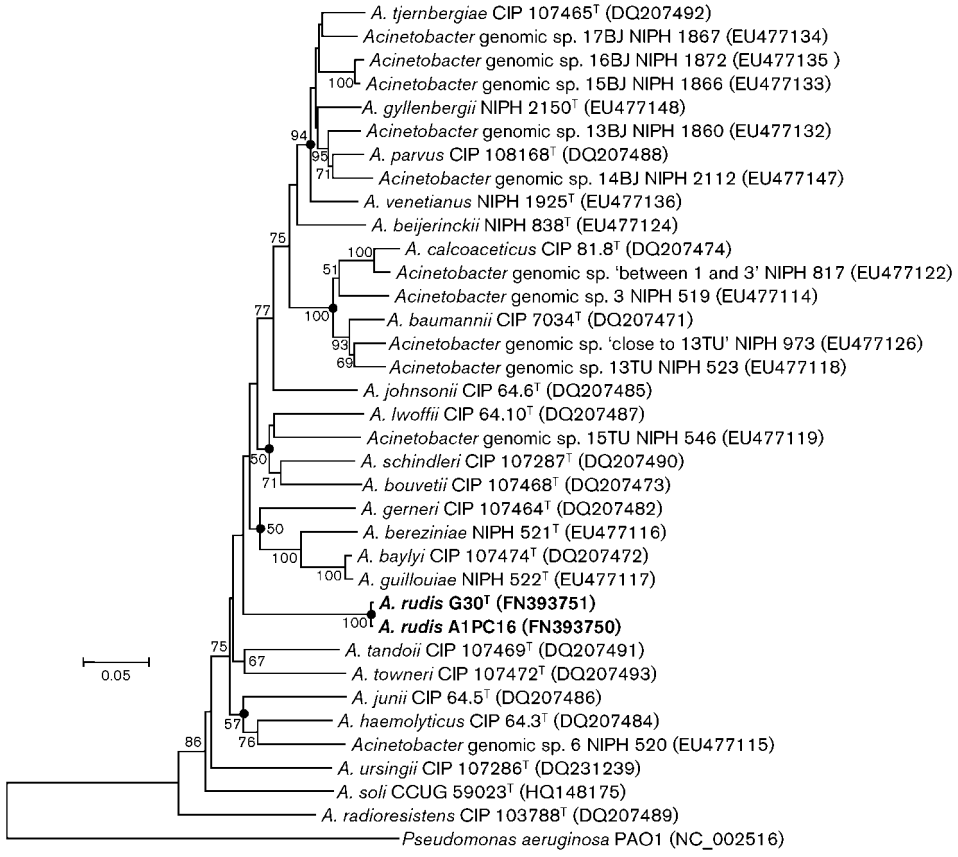

Fig. 2. Rooted tree based on concatenated Z1–Z2 regions of the *rpoB* gene, showing the nearest neighbours of strains G30^T and A1PC16. The tree was generated by the neighbour-joining method. The sequence of *Pseudomonas aeruginosa* PAO1 was used as an outgroup. Bootstrap percentages, generated from 1000 resamplings, $\geqslant$50 % are indicated at branch points. Filled circles indicate branches also recovered by the maximum-parsimony method. Bar, 5 substitutions per 100 nucleotide positions.

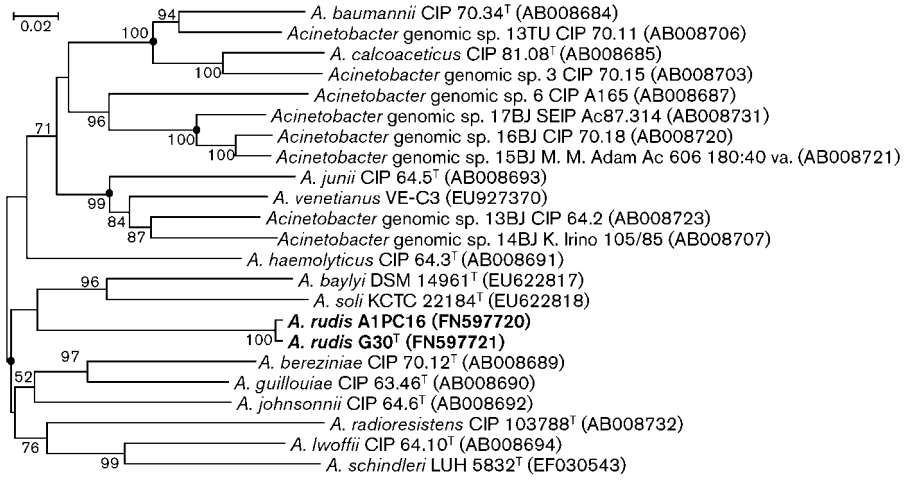

Fig. 3. Unrooted tree based on *gyrB* gene sequences, showing the nearest neighbours of strains G30^T and A1PC16. Bootstrap percentages, generated from 1000 resamplings, $\geqslant$50 % are indicated at branch points. Filled circles indicate branches also recovered by the maximum-parsimony method. Bar, 2 substitutions per 100 nucleotide positions.

Table 1. Phenotypic characteristics that differentiate strains G30^T and A1PC16 from type strains of related species

Strains: 1, G30^T; 2, A1PC16; 3, *A. baumannii* CCUG 19096^T; 4, *A. baylyi* CCUG 50765^T; 5, *A. bereziniae* LMG 1003^T; 6, *A. calcoaceticus* CCUG 12804^T; 7, *A. guillouiae* LMG 988^T; 8, *A. johnsonii* CCUG 19095^T; 9, *A. junii* CCUG 889^T; 10, *A. radioresistens* CCUG 56440^T; 11, *A. venetianus* CCUG 45561^T. All strains were tested in parallel in this study. None of the following sole carbon sources was assimilated by any of the tested strains: L-rhamnose, *N*-acetylglucosamine, inositol, sucrose, maltose, itaconic acid, potassium 2-ketogluconate, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, L-serine, D-mannitol, salicin, melibiose, L-fucose and D-sorbitol. All strains assimilated sodium acetate. W, Weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth at 41 °C	−	−	+	−	−	−	−	−	+	−	−
Acid from glucose	−	−	+	+	+*	−†	−	−	−	−	−
Assimilation of:											
Adipate	−	−	+	+	+*	+	+	−	−	+*	−
L-Alanine	+	+	+	+	+	+	+	+	+	−	+
L-Arginine	−	−	+	−†	−	+	−	+*	+	+	+
L-Arabinose	−	−	+	−	−	−*	−	−	−	−	−
L-Aspartate	−	−	+	+	+	+	+	+*	+*	−	−
2,3-Butanediol	+	+	+	+	+	+	+	−*	−	−	−
Capric acid‡	+	+	+	+	+	−	W	+	+	−	+
Citrate	+	W	+	+	+	−	+	−	+	−	+
Ethanol	+	+	+	+	+	+	+	+	+*	−†	+
D-Glucose	−	−	−	+	−	−	−	−	−	−	−
Histamine	−	−	−	−	−*	−	+*	−	−	−	−
4-Hydroxybenzoic acid	+	+	+	+	+*	+	+*	+*	−	−	−
3-Hydroxybutyric acid‡	+	+	+	−	+	+	+	+	+	−	−
Lactic acid	+	+	+	+	+	+	+	+	+	+	−
L-Leucine	W	W	+	−	−	−*	−	−	−*	+	+
Phenylacetate	+	+	+	−	−*	−†	+*	−	−	−	−
L-Phenylalanine	+	+	+	−	−	+	−	−	−	W*	−
L-Proline	−	−	+	+	+	+	+	+	+	+	+
Propionic acid‡	+	+	+	+	+	W	+	+	+	−	+
Putrescine	−	−	+	−	−	+	−	−	−	+	−
D-Ribose	−	−	+	−	−	−	−	−	−	−	−
Sodium benzoate	+	+	+	+	+	−	+	+	+	−†	+
Sodium malonate	+	+	+	+	−	−†	−*	−*	−	+	+
Suberic acid	−	−	+	+	+	+	+	−	−	+	−
Valeric acid‡	+	+	+	+	+	+	+	+	+	−	+

*According to the literature, variable results for this test may be observed for members of this species.
†Result different from that described in the literature.
‡Not tested previously in other studies.

and the absence of the fatty acids $C_{17:1}\omega8c$ and $C_{17:0}$; from *A. guillouiae* LMG 988^T, assimilation of adipate, L-aspartate, L-phenylalanine and suberic acid and the proportions of the fatty acids $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{18:1}\omega7c$; from *A. bereziniae* LMG 1003^T, assimilation of L-aspartate, L-leucine, L-phenylalanine, suberic acid and sodium malonate and lower proportions of the fatty acids $C_{18:1}\omega9c$ and $C_{18:1}\omega7c$. Other distinctive traits between strains G30^T and A1PC16 and the type strains of closely related neighbours examined in this study are described in Table 1 and Supplementary Table S1.

The phenotypic traits determined in this study were, in general, in agreement with previous publications (Supplementary Table S2). On the basis of information obtained in the current study and in the literature for the 22 species of the genus *Acinetobacter* with validly published names, it is possible to suggest a path of identification of the proposed novel species. Thus, members of the proposed novel species can be identified (i.e. distinguished from all species of the genus with validly published names) as non-haemolytic, with the ability to assimilate 4-hydroxybenzoate, malonate, phenylacetate, L-histidine, L-leucine and phenylalanine, but not adipate, putrescine or L-aspartate, as single carbon sources. Together, the arguments stated above support the proposal of a novel species within the genus *Acinetobacter*, named *Acinetobacter rudis* sp. nov., represented by strains G30^T and A1PC16.

Description of *Acinetobacter rudis* sp. nov.

Acinetobacter rudis (ru′dis. L. adj. *rudis*, *-is* raw, not processed; N.L. gen. n. *rudis* of/from raw unprocessed products).

Colonies are white, circular, convex and smooth after 24 h of incubation on PCA. Cells are Gram-negative, strictly aerobic, non-motile coccobacilli (0.56±0.10 μm diameter). Catalase-positive and oxidase-negative. Growth occurs at 10–37 °C, pH 5.0–9.5 and in the presence of up to 3 % NaCl. After 7 days of incubation, no growth is observed at 6 or 40 °C or in the presence of 5 % NaCl. Growth does not occur in an anaerobic atmosphere and nitrate is not reduced. Haemolysis is not observed. Utilization of Simmons' citrate (growth) is positive. Aesculin is not hydrolysed. H_2S and indole are not produced. Glucose is not fermented. Acid is not produced from glucose. Produces the enzymes alkaline and acid phosphatase, esterase C4, esterase lipase C8, leucine arylamidase and naphthol-AS-BI-phosphohydrolase; produces valine and cystine arylamidases weakly. The enzymes α- and β-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not produced. Sole carbon sources assimilated include L-malate, citrate, phenylacetate, sodium malonate, sodium acetate, L-alanine, L-histidine, ethanol, sodium benzoate, 2,3-butanediol, L-phenylalanine and propionic, lactic, capric, valeric, 3-hydroxybutyric and 4-hydroxybenzoic acids. L-Leucine is assimilated weakly after 48 h of incubation. Carbon sources not assimilated include D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, adipate, L-rhamnose, D-ribose, inositol, sucrose, itaconic acid, suberic acid, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, L-serine, salicin, melibiose, L-fucose, D-sorbitol, potassium 2-ketogluconate, L-proline, L-arginine, putrescine, L-aspartate and histamine. The predominant cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH and the major respiratory quinones are ubiquinones Q-9 and Q-8. The genomic DNA G+C content is 39–40 mol%.

Strain G30^T (=LMG 26107^T =CCUG 57889^T =DSM 24031^T =CECT 7818^T) is the type strain, isolated from raw milk.

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