Acinetobacter parvus sp. nov., a small-colony-forming species isolated from human clinical specimens

Alexandr Nemec,[1] Lenie Dijkshoorn,[2] Ilse Cleenwerck,[3] Thierry De Baere,[4] Danielle Janssens,[3] Tanny J. K. van der Reijden,[2] Petr Ježek[5] and Mario Vaneechoutte[4]

Correspondence
Alexandr Nemec
anemec@szu.cz

[1]National Institute of Public Health, Šrobárova 48, 100 42 Prague, Czech Republic

[2]Department of Infectious Diseases, Leiden University Medical Center C5-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands

[3]BCCM/LMG Bacteria Collection, University of Ghent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

[4]Department of Clinical Chemistry, Microbiology and Immunology, University Hospital, Blok A, B-9000 Gent, Belgium

[5]Department of Clinical Microbiology, U nemocnice 85, Příbram, Czech Republic

The taxonomic status of seven glucose-non-acidifying, non-proteolytic *Acinetobacter* strains characterized by forming small colonies on agar media was studied. With one exception, all strains were from human specimens. They could be distinguished from all described *Acinetobacter* (genomic) species by their ability to grow on ethanol and acetate as sole sources of carbon but not on 22 other substrates tested including DL-lactate or DL-4-aminobutyrate. DNA–DNA hybridization studies, 16S rRNA gene sequence analysis, amplified rDNA restriction analysis and DNA polymorphism analysis by AFLP showed that these strains represent a hitherto unknown species of the genus *Acinetobacter*, for which the name *Acinetobacter parvus* (type strain LMG 21765^T = LUH 4616^T = NIPH 384^T = CCM 7030^T) is proposed.

The genus *Acinetobacter* comprises non-motile, strictly aerobic, oxidase-negative, Gram-negative bacteria that grow well on simple media. Twenty-four (genomic) species are currently recognized within the genus (Bouvet & Grimont, 1986; Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993; Vaneechoutte *et al.*, 1999; Nemec *et al.*, 2001) and strains of these species usually form colonies of 1·0–2·0 mm in diameter after 24 h incubation under optimum growth conditions (Bouvet & Grimont, 1986; Nemec *et al.*, 2001). In a taxonomic study of *Acinetobacter* clinical isolates (Nemec *et al.*, 2000), two strains were found which formed notably small colonies on routine agar media and could not be identified as any known (genomic) species. These strains were glucose-non-acidifying, non-proteolytic, did not utilize any of the 14 carbon sources of the identification scheme of Bouvet & Grimont (1987) and had highly similar amplified rDNA restriction analysis (ARDRA) profiles. Later, five strains similar to the two strains were found among archive strains in our collections. The aim of the present study was to define the taxonomic status of these strains by a polyphasic analysis.

The seven strains used in this study are listed in Table 1. All had the properties of the genus *Acinetobacter* (Juni, 1984), i.e. they were Gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli, and were positive in the transformation assay of Juni (1972). The methods for genotypic characterization included ARDRA, AFLP fingerprinting and comparative 16S rDNA sequence analysis. Phenotypic characterization was done essentially according to Bouvet & Grimont (1987) and Gerner-Smidt *et al.* (1991), with some modifications. Details of the methods and their interpretative criteria have been given by Dijkshoorn *et al.* (1998) and Nemec *et al.* (2000, 2001). The assimilation tests were performed in tubes containing the fluid medium of Cruze *et al.* (1979) supplemented with 0·1 % (w/v) carbon source. Results were read after 2, 6 and 10 days incubation at 30 °C.

High-molecular-mass DNA for determination of the G+C

Published online ahead of print on 20 June 2003 as DOI 10.1099/ijs.0.02631-0.

Abbreviation: ARDRA, amplified rDNA restriction analysis.

The EMBL accession numbers for the 16S rRNA gene sequences of *Acinetobacter parvus* LMG 21765^T and LMG 21766 are AJ293691 and AJ293690, respectively.

Table 1. Strains of *Acinetobacter parvus* used in this study

CCM, Czech Collection of Microorganisms, Brno, Czech Republic; LMG, Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium; LUH and RUH, Collection L. Dijkshoorn, Leiden University Medical Center, Leiden, The Netherlands; NIPH, Collection A. Nemec, National Institute of Public Health, Prague, Czech Republic; CZ, Czech Republic; NL, The Netherlands.

Strain designation	Other strain designation	Reference/received from	Specimen	Location and year of isolation
LMG 21765^T	LUH 4616^T = NIPH 384^T = CCM 7030^T	Nemec *et al.* (2000)	Ear (outpatient)	Příbram, CZ, 1996
LUH 4619	NIPH 399 = CCM 7073	Nemec *et al.* (2000)	Eye (outpatient)	Příbram, CZ, 1996
LUH 3067	–	A. T. Bernards	Forehead (inpatient)	Enschede, NL, 1995
LMG 21766	LUH 3313	Bernards *et al.* (1997)	Skin (inpatient)	Leiden, NL, 1995
RUH 2008	–	–	Blood (inpatient)	Rotterdam, NL, 1986
RUH 2714	–	–	Eye (man)	Rotterdam, NL, 1988
LUH 7036	V0102891	J. Wagenaar	Ear (dog)	Leiderdorp, NL, 2001

content and for DNA–DNA hybridization was prepared from cells grown aerobically on Tryptone Soya Agar (TSA; Oxoid) at 28 °C by the method of Wilson (1987), with minor modifications. Strains *Acinetobacter haemolyticus* LMG 996^T, *Acinetobacter baumannii* LMG 1041^T, *Acinetobacter calcoaceticus* LMG 1046^T and '*Acinetobacter venetianus*' LMG 19082, which produced large amounts of exopolysaccharides, were subjected to a mild alkaline hydrolysis step before cell lysis, as described by Willems *et al.* (2001). The G+C content of the DNA was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage λ DNA (Sigma) was used as the calibration reference. DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) and Goris *et al.* (1998). Hybridizations were performed at 37 °C in a hybridization solution [2 × SSC, 5 × Denhardt's solution, 50 % (v/v) formamide, 2·5 % (w/v) dextran sulfate, low-molecular-mass denatured salmon sperm DNA to a final concentration of 100 μg ml^{-1}, 1·25 μg biotinylated probe DNA ml^{-1}]. The DNA–DNA relatedness percentages presented are means based on at least two hybridization experiments. Reciprocal reactions (e.g. AxB and BxA) were performed and the variation between them was within the limit of this method (Goris *et al.*, 1998).

Colonies of all strains grown on TSA or Nutrient Agar (NA; Oxoid) were circular, convex, smooth and slightly opaque with entire margins. These colonies were notably smaller than those of the other described *Acinetobacter* species (Fig. 1). On NA, the colonies were 0·1–0·4 mm and 0·3–0·9 mm in diameter after 24 and 48 h incubation at 30 °C, respectively, while on TSA, the colonies were 0·3–0·7 mm and 1·0–1·4 mm in diameter after 24 and 48 h of incubation at 30 °C, respectively. The use of other agar media including chocolate and blood agar did not significantly affect colony size as compared with TSA.

All strains grew in Brain–Heart Infusion (Difco) broth at temperatures ranging from 25 to 35 °C but not at 41 °C. All but one strain (LMG 21766) grew at 37 °C, although the growth of LUH 3067 and RUH 2008 was reduced at this temperature as compared to growth at 30 °C. All strains utilized ethanol and acetate as sole sources of carbon and energy and their growth on these two substrates was clear after 2 days incubation. All strains were negative in the following tests: acid production from D-glucose, haemolysis of sheep blood, gelatinase production, and the utilization of DL-lactate, DL-4-aminobutyrate, *trans*-aconitate, citrate (Simmons), glutarate, L-aspartate, azelate, β-alanine, L-histidine, D-malate, malonate, histamine, L-phenylalanine, phenylacetate, levulinate, citraconate, 4-hydroxybenzoate, L-tartrate, L-ornithine, L-leucine, L-arabinose and 2,3-butanediol.

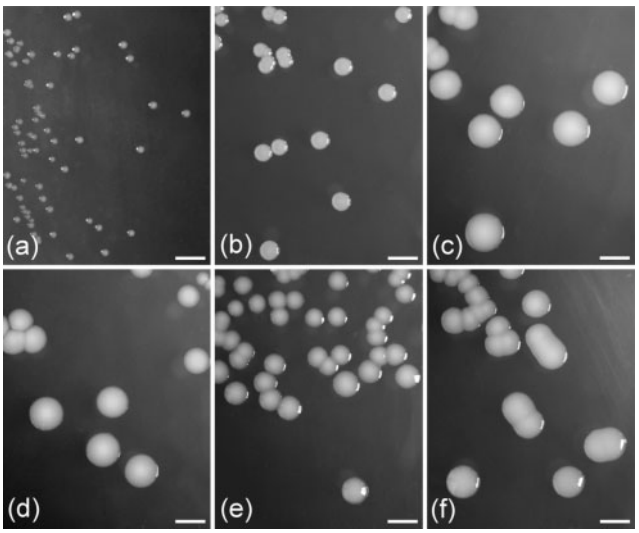

Fig. 1. Colonies of *Acinetobacter parvus* and the type strains of selected *Acinetobacter* species. (a) *A. parvus* LMG 21765^T; (b) *Acinetobacter ursingii* LUH 3792^T; (c) *Acinetobacter lwoffii* ATCC 15309^T; (d) *A. johnsonii* ATCC 17909^T; (e) *A. junii* ATCC 17908^T; (f) *Acinetobacter schindleri* LUH 5832^T. The strains were grown on TSA at 30 °C for 24 h. Bars, 2 mm.

The result of the comparative analysis of AFLP patterns of

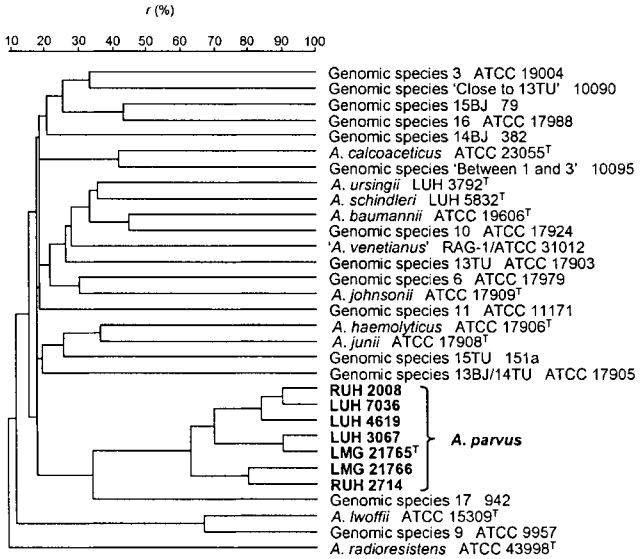

Fig. 2. Dendrogram of cluster analysis of AFLP fingerprints of seven strains of *Acinetobacter parvus* and 24 strains representing all known (genomic) species of the genus *Acinetobacter*. Fingerprints were generated using automated laser fluorescence detection and cluster analysis was performed with the BIONUMERICS software package (Applied Maths) using Pearson's product for similarity calculation and UPGMA for clustering (Nemec *et al.*, 2001).

the seven strains and type and reference strains of all described *Acinetobacter* (genomic) species is shown in Fig. 2. The seven strains grouped at 63 %, which is well above the 50 % level seen in previous studies for the delineation of *Acinetobacter* species (Nemec *et al.*, 2001). They were clearly separated from the other *Acinetobacter* (genomic) species (each species represented by one strain) at 34 %.

The 16S rDNA sequences of strains LMG 21765^T and LMG 21766 (EMBL accession nos AJ293691 and AJ293690, respectively) showed 99·8 % similarity. The similarity values between these sequences and those of the other 24 (genomic) species of the genus *Acinetobacter* (EMBL accession nos Z93434–Z93454, AJ275038, AJ278311 and AJ295007) were in the range of 95·9–98·1 %, which corresponds to the interspecies similarity values of the genus *Acinetobacter* (Ibrahim *et al.*, 1997; Nemec *et al.*, 2001).

Structural homogeneity of 16S rDNA was confirmed by ARDRA. All strains had identical or almost identical restriction patterns: *Cfo*I 1 (LMG 21765^T, LUH 3067, LMG 21766 and RUH 2714) or *Cfo*I 1 + 5 (RUH 2008, LUH 4619 and LUH 7036), *Alu*I 2, *Mbo*I 1, *Rsa*I 2 and *Msp*I 3.

DNA–DNA relatedness was determined between LMG 21765^T, LMG 21766 and the type strains of the nomenspecies that had shown highest similarity (> 96·5 %) of 16S rDNA sequences with the two strains (Table 2). The level of

Table 2. DNA–DNA binding values (%) between LMG 21765^T, LMG 21766 and strains of related *Acinetobacter* species

Strain	LMG 21765^T	LMG 21766
A. parvus LMG 21765^T	100	82
A. parvus LMG 21766	82	100
A. junii LMG 998^T	32	33
A. haemolyticus LMG 996^T	35	30
A. baumannii LMG 1041^T	21	20
A. johnsonii LMG 999^T	22	20
A. calcoaceticus LMG 1046^T	18	19
'*A. venetianus*' LMG 19082/RAG-1	29	29

DNA–DNA binding between LMG 21765^T and LMG 21766 was 82 %. DNA–DNA binding values between these strains and the type strains of *Acinetobacter junii*, *A. haemolyticus*, *A. baumannii*, *Acinetobacter johnsonii*, *A. calcoaceticus* and the reference strain of '*A. venetianus*' were not higher than 35 %. The DNA G+C content of LMG 21765^T and LMG 21766 was 41·8 and 41·5 %, respectively.

On the basis of phenotypic and genotypic characteristics, it is proposed that the seven small-colony-forming strains represent a hitherto unknown species of the genus *Acinetobacter*, for which the name *Acinetobacter parvus* is proposed.

A. parvus can be differentiated from other *Acinetobacter* (genomic) species by its negative results in biochemical tests suggested by Bouvet & Grimont (1987), in particular by the inability to oxidize D-glucose, to hydrolyse gelatin and to utilize DL-lactate, DL-4-aminobutyrate, citrate (Simmons), azelate, *β*-alanine and L-histidine (Bouvet & Grimont, 1987; Bouvet & Jeanjean, 1989; Gerner-Smidt *et al.*, 1991; Vaneechoutte *et al.*, 1999; Nemec *et al.*, 2001). The acetate utilization test which is positive in *A. parvus* is necessary to differentiate prototrophic *A. parvus* strains from auxotrophic strains of other *Acinetobacter* (genomic) species. Notably, its typical colony size is an important feature to recognize *A. parvus* amidst colonies of other species and genera, and to differentiate it from biochemically inactive strains of other *Acinetobacter* (genomic) species.

ARDRA allowed for differentiation of *A. parvus* from all described (genomic) species of *Acinetobacter*, except *A. junii* and proteolytic genomic species 17 (Dijkshoorn *et al.*, 1998; Vaneechoutte *et al.*, 1999; Nemec *et al.*, 2001). Four *A. parvus* strains had the same ARDRA combination pattern (*Cfo*I 1, *Alu*I 2, *Mbo*I 1, *Rsa*I 2, *Msp*I 3) as the latter two (genomic) species. However, *A. parvus* strains can easily be distinguished from genomic species 17 and *A. junii* strains sharing this ARDRA profile by their small colonies and the inability to lyse sheep erythrocytes.

The *A. parvus* strains were isolated from human and animal non-sterile body sites, except for RUH 2008, which originated from the blood of a human. Isolation of this strain was

followed by other isolates with similar characteristics from intravenous catheters, which indicates that the strain was involved in a catheter-related blood-stream infection. Strain LUH 7036 was isolated from the ear of a dog with refractory otitis media.

During this study, an additional strain (LUH 4826) that was phenotypically indistinguishable from the *A. parvus* strains was isolated from a human clinical specimen. However, LUH 4826 had *Alu*I and *Rsa*I ARDRA patterns different from those of the *A. parvus* strains and AFLP fingerprinting showed no significant similarity between this strain and any of the described *Acinetobacter* (genomic) species including *A. parvus* (not shown). Therefore, LUH 4826 may represent an as-yet-undescribed species of the genus *Acinetobacter* that is phenotypically similar to *A. parvus*. This finding demonstrates that, as is the case with most *Acinetobacter* (genomic) species, definitive species identification requires the use of genotypic methods.

Description of *Acinetobacter parvus* sp. nov.

Acinetobacter parvus (par′vus. L. masc. adj. *parvus* small, referring to the fact that its colonies on agar media are significantly smaller than those of the other known *Acinetobacter* species).

Characteristics correspond to those of the genus (Juni, 1984). The description is based on the characterization of seven strains of different origin. Colonies on TSA after 24 h incubation at 30 °C are approximately 0·3–0·7 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs at 35 °C but not at 41 °C. Growth at 37 °C usually occurs but may be reduced. Good growth on ethanol and acetate as sole sources of carbon and energy. Negative results in the following tests: acid production from D-glucose, haemolysis of sheep blood, gelatinase production and the utilization of DL-lactate, DL-4-aminobutyrate, *trans*-aconitate, citrate (Simmons), glutarate, L-aspartate, azelate, *β*-alanine, L-histidine, D-malate, malonate, histamine, L-phenylalanine, phenylacetate, levulinate, citraconate, 4-hydroxybenzoate, L-tartrate, L-ornithine, L-leucine, L-arabinose and 2,3-butanediol.

The type strain is LMG 21765^T (=LUH 4616^T=NIPH 384^T=CCM 7030^T). Isolated from the ear of an outpatient. This strain grows well at 37 °C and has the following restriction patterns of amplified 16S rDNA: *Cfo*I 1, *Alu*I 2, *Mbo*I 1, *Rsa*I 2, *Msp*I 3. Its DNA G+C content is 41·8 %.

Acknowledgements

We thank Dr A. T. Bernards (Leiden University Medical Center) and Dr J. Wagenaar (ID-Lelystad) for generous provision of strains.

Note added in proof

Since the study was completed, seven additional strains with typical *A. parvus* colonies have been studied in our laboratories. All of them were isolated from human clinical specimens (blood, ear pus, vaginal swab) and showed AFLP fingerprints, ARDRA profiles and biochemical properties typical of *A. parvus*. The only exception was the ability of three of these strains to grow on L-ornithine. Since this article was accepted for publication, seven new species of *Acinetobacter* have been described (Carr *et al.*, 2003). Comparison of published 16S rDNA sequences and phenotypic characteristics did not show the identity of *A. parvus* with any of these species.

References

Bernards, A. T., de Beaufort, A. J., Dijkshoorn, L. & van Boven, C. P. A. (1997). Outbreak of septicaemia in neonates caused by *Acinetobacter junii* investigated by amplified ribosomal DNA restriction analysis (ARDRA) and four typing methods. *J Hosp Infect* **35**, 129–140.

Bouvet, P. J. M. & Grimont, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol* **36**, 228–240.

Bouvet, P. J. M. & Grimont, P. A. D. (1987). Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann Inst Pasteur Microbiol* **138**, 569–578.

Bouvet, P. J. M. & Jeanjean, S. (1989). Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Res Microbiol* **140**, 291–299.

Carr, E. L., Kämpfer, P., Patel, B. K. C., Gürtler, V. & Seviour, R. J. (2003). Seven novel species of *Acinetobacter* isolated from activated sludge. *Int J Syst Evol Microbiol* **53**, 953–963.

Cruze, J. A., Singer, J. T. & Finnerty, W. R. (1979). Conditions for quantitative transformation in *Acinetobacter calcoaceticus*. *Curr Microbiol* **3**, 129–132.

Dijkshoorn, L., van Harsselaar, B., Tjernberg, I., Bouvet, P. J. M. & Vaneechoutte, M. (1998). Evaluation of amplified ribosomal DNA restriction analysis for identification of *Acinetobacter* genomic species. *Syst Appl Microbiol* **21**, 33–39.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Gerner-Smidt, P. & Tjernberg, I. (1993). *Acinetobacter* in Denmark: II. Molecular studies of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *APMIS* **101**, 826–832.

Gerner-Smidt, P., Tjernberg, I. & Ursing, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *J Clin Microbiol* **29**, 277–282.

Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.

Ibrahim, A., Gerner-Smidt, P. & Liesack, W. (1997). Phylogenetic relationship of the twenty-one DNA groups of the genus *Acinetobacter* as revealed by 16S ribosomal DNA sequence analysis. *Int J Syst Bacteriol* **47**, 837–841.

Juni, E. (1972). Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J Bacteriol* **112**, 917–931.

Juni, E. (1984). Genus III. *Acinetobacter* Brisou and Prévot 1954, 727^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 303–307. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Nemec, A., Dijkshoorn, L. & Ježek, P. (2000). Recognition of two novel phenons of the genus *Acinetobacter* among non-glucose-acidifying isolates from human specimens. *J Clin Microbiol* **38**, 3937–3941.

Nemec, A., De Baere, T., Tjernberg, I., Vaneechoutte, M., van der Reijden, T. J. K. & Dijkshoorn, L. (2001). *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* **51**, 1891–1899.

Tjernberg, I. & Ursing, J. (1989). Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. *APMIS* **97**, 595–605.

Vaneechoutte, M., Tjernberg, I., Baldi, F., Pepi, M., Fani, R., Sullivan, E. R., van der Toorn, J. & Dijkshoorn, L. (1999). Oil-degrading *Acinetobacter* strain RAG-1 and strains described as '*Acinetobacter venetianus* sp. nov.' belong to the same genomic species. *Res Microbiol* **150**, 69–73.

Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Green Publishing and Wiley-Interscience.