Description of *Acinetobacter venetianus ex* Di Cello *et al.* 1997 sp. nov.

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The name '*Acinetobacter venetianus*' has been used previously to designate three marine hydrocarbon-degrading *Acinetobacter* strains, of which strain RAG-1 (=ATCC 31012) has industrial applications for the production of the bioemulsifier emulsan. However, to date, the name of this taxon has not been validly published. In this study, five strains were examined to corroborate the delineation of this taxon by means of phenotypic characterization, DNA–DNA hybridization, selective restriction fragment amplification (AFLP), amplified rDNA restriction analysis (ARDRA), *rpoB* gene sequence analysis and tRNA intergenic spacer length polymorphism analysis (tDNA-PCR) and to emend the description of '*Acinetobacter venetianus*' (*ex* Di Cello *et al.* 1997). AFLP analysis showed that the five strains formed a tight cluster at 56.8±5.0 % genomic relatedness that was separated from strains of other haemolytic species of the genus *Acinetobacter* and from the type and reference strains of other *Acinetobacter* species at ⩽27 % relatedness, indicating the distinctiveness of the novel strains. The strains were haemolytic and able to grow on citrate (Simmons), L-histidine and malonate. The strains did not oxidize D-glucose or utilize DL-lactate or L-aspartate. The G+C contents of strains RAG-1 and of VE-C3 were 43.9 % and 43.6 mol%, respectively. The novel strains could be recognized by a characteristic ARDRA pattern (*Cfo*I 1, *Alu*I 3, *Mbo*I 2, *Rsa*I 2, *Msp*I 3). The consensus tDNA-PCR pattern for the five strains consisted of amplified fragments of 87.9, 100.2, 134.6 and 248.5 bp and was indistinguishable from that of strains of *Acinetobacter* genomic species 14BJ. The five strains represent a novel species for which the name *Acinetobacter venetianus* sp. nov. is proposed. The type strain is RAG-1^T (=ATCC 31012^T=CCUG 45561^T=LMG 19082^T=LUH 3904^T=NIPH 1925^T).

Abbreviations: AFLP, selective restriction fragment amplification; ARDRA, amplified rRNA restriction analysis; tDNA-PCR, intergenic tRNA spacer length polymorphism analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are AM909651 and EU258608–EU258610. The accession numbers for the *rpoB* partial gene sequences are EU477136 and EU496378–EU496381.

A supplementary figure showing the growth of different *Acinetobacter* strains after 72 h in mineral medium containing carbon sources of variable chain lengths and a supplementary table showing the *P*-values of the differences in delta yield over the range of carbon sources used between various *Acinetobacter* strains and strain RAG-1 are available with the online version of this paper.

Acinetobacter strain RAG-1 (=ATCC 31012) was isolated from seawater near a beach (Tel Baruch, Israel) after enrichment of mineral medium with sterilized crude paraffinic oil (Reisfeld *et al.*, 1972; Rosenberg *et al.*, 1979). This strain is industrially important since it produces a potent bioemulsifier (emulsan) (Sar & Rosenberg, 1983; Shabtai & Gutnick, 1985; Shabtai, 1990; Gutnick *et al.*, 1991). Strain RAG-1 was originally identified as a member of the genus *Arthrobacter* (Reisfeld *et al.*, 1972), but has been most often referred to as *Acinetobacter lwoffii* (e.g. Alon & Gutnick, 1993) or *Acinetobacter calcoaceticus* (Sar & Rosenberg, 1983; Leahy *et al.*, 1993).

More recently, strains of the genus *Acinetobacter* were isolated from a bacterial oil-degrading consortium from the Venice lagoon (Italy) and were shown to be capable of metabolizing fuel oil. These strains were named '*Acinetobacter venetianus*' (Di Cello *et al.*, 1997). Strain VE-C3 of this consortium was shown to have the same amplified rDNA restriction analysis (ARDRA) pattern (Vaneechoutte *et al.*, 1995; Dijkshoorn *et al.*, 1998) as strain RAG-1 (Vaneechoutte *et al.*, 1999). In addition, Yamamoto & Harayama (1996) reported that an n-tetradecane-degrading strain (T4) from the Japanese Sea shared 98.8 % *gyrB* gene sequence similarity with strain RAG-1. The close relatedness of the three strains was also confirmed by analysis of the nucleotide sequence (GenBank accession no. EU927370) of a 873 bp *gyrB* gene fragment obtained via PCR from the genome of strain VE-C3. It was found that strain VE-C3 shared the highest *gyrB* gene sequence similarity (97 %) with strains T4 and RAG-1 (Baldi *et al.*, 2001) and had an amino acid-based sequence similarity of 99.3 %. In addition, strain T4 had an ARDRA pattern identical to that of strain RAG-1 (Vaneechoutte *et al.*, 1999). In this study, data for two additional strains with the same characteristic ARDRA pattern are presented. One of the strains was isolated from the water purification system of an aquaculture pond in Denmark (LUH 5627) and the other strain was isolated from a vegetable market in Hong Kong (LUH 7437). Phenotypic and genotypic data of the five currently available strains (Table 1), including data obtained previously for strains RAG-1, T4 and VE-C3 (Vaneechoutte *et al.*, 1999), show that these organisms should be considered as a novel species for which we propose the previously used name '*Acinetobacter venetianus*' (*ex* Di Cello *et al.* 1997).

Strain RAG-1 was previously assigned to the genus *Acinetobacter* by means of Juni's transformation test (Juni, 1984) in which DNA of strain RAG-1 transformed the *Acinetobacter baylyi* mutant strain BD413 to prototrophy (Kaplan & Rosenberg, 1982).

It has also been shown previously that the DNA G+C contents of strains RAG-1 and VE-C3 are 43.9 % and 43.6 mol%, respectively (Vaneechoutte *et al.*, 1999). These values fall within the range of 38–47 % expected for members of the genus *Acinetobacter* (Juni, 1984).

Furthermore, DNA–DNA hybridization studies, conducted using a previously described filter method (Tjernberg *et al.*, 1989), have already indicated that strains RAG-1 and VE-C3 belong to a unique genomic species (Vaneechoutte *et al.*, 1999). The two strains differed by only 0.4 °C in the melting temperature value, well below the 3 °C limit used as the species delineation threshold for this method. Moreover, Tjernberg *et al.* (1989) showed that both strains were different from the 18 *Acinetobacter* genomic species described at the time and from strains belonging to the *Acinetobacter* genomic species 'between 1 and 3' and 'close to 13TU' (Gerner-Smidt & Tjernberg, 1993).

For the present study, phenotypic analyses were performed as described by Nemec *et al.* (2009) and a high degree of similarity was found between the biochemical properties of all five strains. All five strains showed haemolytic activity on Columbia agar with 5 % sheep blood (bioMérieux) at 30 and 37 °C and all, except strain LUH 7437, were able to hydrolyse gelatin. None of the strains produced acid from D-glucose. When inoculated into Brain Heart Infusion broth (Oxoid), all five strains showed clear signs of growth after one day at 37 °C, but not after two days incubation at 41 °C. In assimilation tests, all five strains utilized acetate, 4-aminobutyrate, L-arginine, benzoate, citrate (Simmons), ethanol, L-histidine, L-leucine, D-malate and malonate with

Table 1. '*Acinetobacter venetianus*' strain designations, origins, donors and sequence accession numbers

Strain designations*	Origin	Donor	GenBank accession no.	
			16S rRNA gene	*rpoB*
RAG-1 (=ATCC 31012=CCUG 45561=LMG 19082=LUH 3904 =NIPH 1925)	Seawater, Tel Baruch, Israel	D. Gutnick	AJ295007 (AVE295007)	EU477136
LUH 4379 (=VE-C3)	Oil in Venice lagoon, Adriatic Sea, Italy	F. Baldi	AM909651	EU496378
LUH 5627 (=S1-2)	Aquaculture pond, Denmark	L. Guardabassi	EU258608	EU496381
LUH 7437 (=CUHK 7025)	Vegetable market, Hong Kong	E. Houang	EU258610	EU496380
LUH 8758 (=T4=MBIC 1332)	Japanese Sea	S. Yamamoto	EU258609	EU496379

*CUHK, Chinese University of Hong Kong Culture Collection, Hong Kong, China; LUH, Collection Leiden University Medical Center, Leiden, the Netherlands; NIPH, Collection of A. Nemec, National Institute for Public Health, Prague, Czech Republic.

clear positive reactions evident after four (mostly in two) days incubation. None of the five strains grew on *trans*-aconitate, *β*-alanine, L-arabinose, L-aspartate, 2,3-butanediol, citraconate, D-glucose, D-gluconate, gentisate, glutarate, histamine, 4-hydroxybenzoate, DL-lactate, levulinate, L-ornithine, phenylacetate, L-phenylalanine, putrescine, L-tartrate, tricarballylate, trigonelline or tryptamine within 10 days. Only strain T4 was able to grow on azelate and adipate. Thus, the five '*Acinetobacter venetianus*' strains could be differentiated from species of the genus *Acinetobacter* that comprise only non-haemolytic strains, i.e. *Acinetobacter baumannii*, *Acinetobacter baylyi*, *Acinetobacter bouvetii*, *Acinetobacter calcoaceticus*, *Acinetobacter gerneri*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *Acinetobacter parvus*, *Acinetobacter schindleri*, *Acinetobacter tandoii*, *Acinetobacter towneri*, *Acinetobacter ursingii* and *Acinetobacter* genomic species 3, 10, 11, 13TU and 15TU, on the basis of their haemolytic activity and other biochemical tests. In particular, the ability of the five '*Acinetobacter venetianus*' strains to grow on citrate (Simmons), L-histidine and malonate, and their inability to oxidize D-glucose and to utilize DL-lactate and L-aspartate (Bouvet & Grimont, 1987; Gerner-Smidt *et al.*, 1991; Vaneechoutte *et al.*, 1999; Nemec *et al.*, 2001, 2003; Carr *et al.*, 2003) were important distinguishing features. The characteristics that are useful for the differentiation of the five strains of '*Acinetobacter venetianus*' from species of the genus *Acinetobacter* that include haemolytic strains are summarized in Table 2. Strains of recognized species were selected for this phenotypic analysis from the studies of Bouvet & Grimont (1986), Tjernberg & Ursing (1989), Dijkshoorn *et al.* (1990), Vaneechoutte *et al.* (1999) and Carr *et al.* (2003). The type strains of the recognized species were used in the analysis. The type strain of *A. tjernbergiae* was also included as it demonstrated haemolysis when tested for this study.

Strikingly, some strains of '*Acinetobacter venetianus*' have been associated with long chain carbohydrate degradation: strain RAG-1 was found to be the only bacterium from a seawater sample that dispersed oil (Reisfeld *et al.*, 1972) and was described as able to degrade hexadecane (Rosenberg, 1984). Strain VE-C3, from an oil spill in the Venice lagoon, was shown to grow on C10, C14 and C20 (Di Cello *et al.*, 1997), and strain T4 from the Japanese Sea was initially described as n-tetradecane-degrading (Yamamoto & Harayama, 1996). In addition, a study of the microbial community on the feathers of two adult male and two adult female Eastern Bluebirds (*Sialis sialis*) from Lee County, Alabama, USA, based on culture as well as cloning of amplified 16S rRNA genes, indicated the presence of '*Acinetobacter venetianus*' on the basis of the sequencing of a partial 16S rRNA gene fragment (GenBank accession no. AJ295007) (Shawkey *et al.*, 2005). Birds

Table 2. Phenotypic characteristics useful for the differentiation of strains of '*Acinetobacter venetianus*' from recognized species of the genus *Acinetobacter* with haemolytic strains and from *Acinetobacter radioresistens*

Taxa: 1, '*Acinetobacter venetianus*'; 2, *Acinetobacter haemolyticus*; 3, *Acinetobacter junii*; 4, *Acinetobacter beijerinckii*; 5, *Acinetobacter gyllenbergii*; 6, *Acinetobacter tjernbergiae*; 7, *Acinetobacter* genomic species 6; 8, *Acinetobacter* genomic species 13BJ/14TU; 9, *Acinetobacter* genomic species 14BJ; 10, *Acinetobacter* genomic species 15BJ; 11, *Acinetobacter* genomic species 16; 12, *Acinetobacter* genomic species 17; 13, *Acinetobacter radioresistens*. +, All strains positive in four days of incubation; −, all strains negative within 10 days of incubation; V, strain-dependent results or weak, delayed or non-reproducible reactions. These results are all from the present study to ensure that the data reported were obtained in the same media and under uniform conditions. To date, no haemolytic strains of *Acinetobacter radioresistens* have been observed, but the species was included because it is closely related to '*Acinetobacter venetianus*' on the basis of 16S rRNA gene sequence.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Number of isolates	5	5	4	15	9	1	2	6	3	2	3	2	6
Acid from:													
D-Glucose	−	V	−	−	−	−	+	+	+	−	−	−	−
Utilization of:													
Adipate	V	−	−	−	+	−	−	−	+	−	−	−	V
β-Alanine	−	−	−	−	+	−	−	V	+	−	V	+	−
4-Aminobutyrate	+	+	V	+	V	−	−	−	+	−	V	+	+
L-Arginine	+	+	+	−	+	+	+	+	+	+	+	+	+
Azelate	V	−	−	−	+	−	−	−	+	−	−	−	+
Benzoate	+	−	+	−	+	+	−	V	+	+	+	+	+
2,3 Butanediol	−	−	−	−	−	−	−	−	−	−	−	−	+
Ethanol	+	V	V	+	V	+	+	−	−	−	−	−	+
L-Histidine	+	+	V	+	+	+	+	+	+	+	+	+	−
4-Hydroxybenzoate	−	+	−	−	V	−	V	V	+	+	V	+	−
DL-Lactate	−	−	+	−	+	−	−	+	+	+	+	+	+
Malonate	+	−	−	+	V	−	−	V	+	V	V	V	+
Putrescine	−	−	−	−	−	−	−	−	+	−	−	+	V

waterproof their feathers using preen oil, which inhibits growth of some bacteria but stimulates the growth of other bacteria (Shawkey *et al.*, 2005). Finally, new strains of the genus *Acinetobacter* have been isolated that can use long chain n-alkanes (Throne-Holst *et al.*, 2006). Therefore, we investigated whether the utilization of alkanes was specific for the five strains of '*Acinetobacter venetianus*'. To this aim, the growth of a series of strains was compared with that of strain RAG-1 using the method of Di Cello *et al.* (1997). Briefly, growth was tested in mineral medium (Mills *et al.*, 1978), enriched with respectively, n-decane (C10), olefine-free n-tetradecane (C14) and n-eicosane (C20) (Fluka). Experiments were performed in quadruplicate for every strain and were compared with the results obtained for strain RAG-1 at the same time point. The protein concentration was used as a measure for growth using the BCA Protein Assay kit (23225; Pierce) with BSA used for calibration. Measurements were performed at 590 nm. Growth results were expressed in (mg protein ml^{-1}) day^{-1}. The statistical analysis is presented in Supplementary Table S1 (available in IJSEM Online). The mean results for all strains with respect to growth in the presence of the various carbon sources are depicted in Supplementary Fig. S1 (IJSEM Online). All strains tested demonstrated significantly ($P<0.01$, except *Acinetobacter junii* RUH 2228^T, *Acinetobacter radioresistens* RUH 2865^T and *Acinetobacter baumannii* RUH 3023^T: $P<0.05$) better growth with increasing chain length. Three of the five strains designated as '*Acinetobacter venetianus*' could efficiently degrade alkanes of various chain lengths, but this feature was not unique for the species.

ARDRA was carried out as described previously (Dijkshoorn *et al.*, 1998; Vaneechoutte *et al.*, 1995). The following restriction patterns were observed for all five strains: *Cfo*I 1, *Alu*I 3, *Mbo*I 2, *Rsa*I 2, *Msp*I 3 [pattern designation according to Dijkshoorn *et al.* (1998)]. This combination of ARDRA patterns has not been observed previously for any of the grouped or ungrouped strains of the Leiden University Medical Center ARDRA profile collection.

For the assessment of the overall genomic relatedness of the five strains, AFLP fingerprinting was performed as described previously (Nemec *et al.*, 2001). The five strains formed a tight cluster at 56.8 ± 5.0 % genomic relatedness which was above the 50 % level considered to be the threshold for the delineation of species of the genus *Acinetobacter* (Nemec *et al.*, 2001, 2003) (data not shown). Furthermore, the five strains were separated from strains of other haemolytic species and from the type and reference strains of other species at $\leqslant 27$ %, which emphasized the genomic coherence and distinctness of the strains designated as members of '*Acinetobacter venetianus*'.

A tDNA-PCR analysis was performed as described previously (Vaneechoutte *et al.*, 1998; Baele *et al.*, 2000). The consensus tDNA-PCR fingerprint for the five strains consisted of amplified tDNA-intergenic spacers with lengths of 87.9, 100.2, 134.6 and 248.5 bp and was indistinguishable from the tDNA-PCR-fingerprints of *Acinetobacter* genomic species 14BJ.

Sequencing of the 16S rRNA gene and comparative analysis were carried out as described previously (Vaneechoutte *et al.*, 2000). The initial designation of the strains as a separate species, named '*Acinetobacter venetianus*' by Di Cello *et al.* (1997), was based solely on the sequence of the 16S rRNA gene and on comparisons with the published 16S rRNA gene sequences of seven species, as available at the time (Rainey *et al.*, 1994). Here, we present a more updated comparison and cluster analysis, including the 16S rRNA gene sequences of all nomenspecies and all recognized DNA groups. This analysis clearly separates the strains designated as '*Acinetobacter venetianus*' from those of the other species (Fig. 1). This enabled us to perform a more extended comparison of '*Acinetobacter venetianus*' (strain VE-C3; GenBank accession number X80285) and revealed between 95 and 97 % gene sequence similarity with the sequences of the 19 other *Acinetobacter* species (Z93434–Z93454), a level indicative of the distinctiveness of '*Acinetobacter venetianus*' (Vaneechoutte & De Baere, 2007).

Analysis of partial sequences of the gene encoding the β-subunit of RNA polymerase (*rpoB*) was performed as described previously (Nemec *et al.*, 2009). The sequences of two variable regions of *rpoB* zones 1 (*rpoB* nucleotide positions 2917–3267) and 2 (positions 3322–3723) were determined for all five strains of '*Acinetobacter venetianus*' and were compared with each other and with previously published sequences (Nemec *et al.*, 2009). The concatenated zones of the five novel strains showed intraspecies similarity values (expressed as the percentage of identical residues derived from pairwise alignments between nucleotide sequences) which ranged from 98.5 to 99.9 %, while the similarity values between the concatenated sequences of the novel strains and those of the other recognized species (Nemec *et al.*, 2009) ranged from 78.4 to 93.6 %. Overall, these data correspond to the inter- and intraspecies similarity values found previously (Nemec *et al.*, 2009) and indicate the close relationship of the five '*Acinetobacter venetianus*' strains to each other and their distinctiveness from hitherto described species of the genus *Acinetobacter*.

Description of *Acinetobacter venetianus* sp. nov.

Acinetobacter venetianus (ve.ne.ti.a′nus. M.L. masc. adj. *venetianus* Venetian, pertaining to Venice, Italy, because one of the strains, VE-C3, was isolated from the Venice lagoon in the Adriatic sea).

Phenotypic characteristics correspond to those of the genus (Baumann *et al.*, 1968), i.e. Gram-negative, strictly aerobic, oxidase-negative, catalase-positive, non-motile coccobacilli, capable of growing in mineral media with acetate as sole carbon source and ammonia as sole source of nitrogen, and incapable of dissimilative denitrification. Positive in the transformation assay of Juni (1972). The species

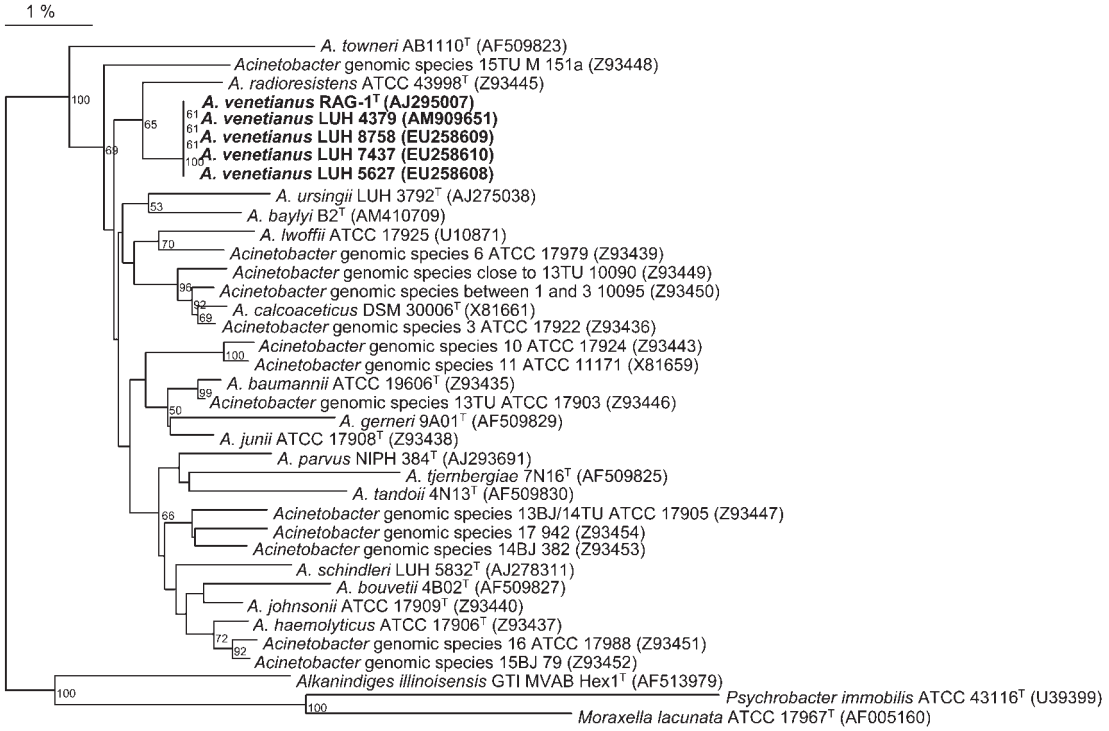

Fig. 1. Rooted tree showing the phylogenetic relationships within the genus *Acinetobacter* on the basis of a region of the 16S rRNA gene sequence corresponding to nucleotides 110–1443 of the *Escherichia coli* gene (GenBank accession no. X80725). The cluster analysis was performed using Genebase (Applied Maths) and the tree was constructed on the basis of the neighbour-joining method using *Psychrobacter immobilis* as an outgroup. Numbers on branches indicate bootstrap values. Bar, 1% sequence divergence. ATCC, American type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; LUH, Leiden University Hospital; NIPH, National Institute Public Health Prague, Czech Republic; RUH, Rijksuniversitair Hospitaal.

description is based on the characterization of five strains of diverse origin (Table 1). Colonies on Columbia agar supplemented with 5% sheep blood after 24 h incubation at 30 °C are ~1.5 mm in diameter, circular, convex, smooth and surrounded by clear zones of haemolysis. The type strain hydrolyses gelatin and does not grow on azelate or adipate. The phenotypic properties are listed in Table 2 and in the results section of the main text. This species is characterized by strains that possess identical biochemical properties except for gelatin hydrolysis and growth on azelate and adipate.

The type strain, RAG-1^T (=ATCC 31012^T=CCUG 45561^T=LMG 19082^T=LUH 3904^T=NIPH 1925^T), was isolated from seawater collected from near a beach (Tel Baruch, Israel) (Reisfeld *et al.*, 1972). The G+C content of the type strain is 43.9 mol%.

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