Acinetobacter gandensis sp. nov. isolated from horse and cattle

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We previously reported the presence of an OXA-23 carbapenemase in an undescribed species of the genus Acinetobacter isolated from horse dung at the Faculty of Veterinary Medicine, Ghent University, Belgium. Here we include six strains to corroborate the delineation of this taxon by phenotypic characterization, DNA–DNA hybridization, 16S rRNA gene and rpoB sequence analysis, % G+C determination, MALDI-TOF MS and fatty acid analysis. The nearly complete 16S rRNA gene sequence of strain UG 60467T showed the highest similarities with those of the type strains of Acinetobacter bouvetii (98.4 %), Acinetobacter haemolyticus (97.7 %), and Acinetobacter schindleri (97.2 %). The partial rpoB sequence of strain UG 60467T showed the highest similarities with 'Acinetobacter bohemicus' ANC 3994 (88.6 %), A. bouvetii NIPH 2281 (88.6 %) and A. schindleri CIP 107287T (87.3 %). Whole-cell MALDI-TOF MS analyses supported the distinctness of the group at the protein level. The predominant fatty acids of strain UG 60467T were C12 : 0 3-OH, C12 : 0, C16 : 0, C18 : 1 ω9c and summed feature 3 (C16 : 1 ω7c and/or iso-C15 : 0 2-OH). Strains UG 60467T and UG 60716 showed a DNA–DNA relatedness of 84 % with each other and a DNA–DNA relatedness with A. schindleri LMG 19576T of 17 % and 20 %, respectively. The DNA G+C content of strain UG 60467T was 39.6 mol%. The name Acinetobacter gandensis sp. nov. is proposed for the novel taxon. The type strain is UG 60467T (=ANC 4275T =LMG 27960T =DSM 28097T).

The genus Acinetobacter was described in 1954 by Brisou & Prévot (1954) and at the time of writing comprises 33 distinct species with validly published names (www.bacterio.net/a/acinetobacter.html). Members of the genus Acinetobacter are Gram-stain-negative coccobacilli, non-motile, strictly aerobic, oxidase-negative, catalase-positive and have the ability to utilize sources of carbon and to grow on minimal salt medium. Some species of the genus Acinetobacter are able to lyse mammalian erythrocytes and liquefy gelatin, while others are usually neither haemolytic nor proteolytic (Vaneechoutte et al., 2011).

Most of the 33 species of the genus Acinetobacter were obtained from human specimens and particularly from colonized or infected patients (Nemec et al., 2000, 2001, 2009, 2011). As a consequence of a high rate use of carbapenems in humans, carbapenem-resistant species of the
genus *Acinetobacter* have been increasingly reported (Poirel & Nordmann, 2006). The most commonly acquired carbapenemases identified among these species correspond to the OXA-type class D β-lactamases (Perichon et al., 2014; Zander et al., 2014). Infections with OXA-23-producing species of the genus *Acinetobacter*, including *Acinetobacter baumannii*, *Acinetobacter johnsonii*, *Acinetobacter pittii* and *Acinetobacter baylyi*, have been reported worldwide (Mugnier et al., 2010; Boo et al., 2009; Zander et al., 2014; Zhou et al., 2011).

OXA-23-producing species of the genus *Acinetobacter* have also been found in non-human environments, such as livestock and pets (Poirel et al., 2012; Guerra et al., 2014, Smet et al., 2012, Vaneechoutte et al., 2000). The presence of carbapenemase-producing species of the genus *Acinetobacter* in animals is worrying, because this may not only complicate treatment of infections in these animals, but may also be a public health hazard.

Recently, we identified six strains belonging to an as-yet-undescribed species of the genus *Acinetobacter*. Three strains, one being an OXA-23 producer, were isolated from the faeces of horses hospitalized at the Faculty of Veterinary Medicine, Ghent University, Belgium (Smet et al., 2012). Three other strains were isolated, one from the mouth of a horse (Tripoli, Lebanon), one from a rectal swab of a cow (Zgharta, Lebanon) and one from water with detritus of a forest pool in a natural reserve (Czech Republic) (Table 1). In the present study, the taxonomic position of these six strains was determined using a polyphasic approach. Based on the genotypic and phenotypic characteristics, these strains were found to represent a novel species of the genus *Acinetobacter*.

To clarify the taxonomic position of the six strains, phylogenetic analysis based on the 16S rRNA gene sequence was performed. The nearly complete 16S rRNA gene sequences of type strains of species belonging to the genus *Acinetobacter* were retrieved from the NCBI Nucleotide database and aligned with the 16S rRNA gene sequences of the six novel strains using the online CLUSTAL Omega Software (http://www.ebi.ac.uk/Tools/msa/clustalo) with default parameters. Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) with the MEGA6.06 software package (http://www.megasoftware.net) (Tamura et al., 2013) and a phylogenetic tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with *Moraxella lacunata* LMG 5301T as the outgroup (Fig. 1).

The results indicate a separate clade of the six novel strains (bootstrap value of 98 %), with ‘*Acinetobacter bohemicus*’ ANC 3994 as the closest neighbour and *Acinetobacter schindleri* LMG 19576T as second closest. The highest sequence similarity with strain UG 60467T was found with *Acinetobacter bouvetii* EU40 (NCBI accession number JF681285.1; 98.4 %).

The *rpoB*-based comparative analysis was performed for an 861 bp region covering nucleotide positions 2915–3775 of the *rpoB* coding region of *A. baumannii* CIP 70.34T as described previously (Nemec et al., 2010, 2011). The *rpoB*-based tree for the six novel strains and for strains representing other species of the genus *Acinetobacter* is depicted in Fig. 2. The intraspecies similarity values (expressed as the percentages of identical nucleotides in corresponding positions in two aligned sequences) for the novel strains were in the range of 99.3–99.9 %, while the similarity values between these strains and the other species ranged from 76.9 % (*Acinetobacter qingfengensis* 2BJ1T) to 88.6 % (*A. bohemicus* ANC 3994 and *A. bouvetii* NIPH 2281T) (NCBI

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### Table 1. Origin and NCBI accession numbers of the six strains of *Acinetobacter gandensis* sp. nov. studied

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Specimen</th>
<th>Locality and date of isolation</th>
<th>NCBI accession no. of 16S rRNA gene sequence</th>
<th>NCBI accession no. of rpoB sequence</th>
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<td>Horse dung from different horses hospitalized in different stables; isolates not associated with the illness for which the horses were hospitalized.</td>
<td>Faculty of Veterinary Medicine, Merelbeke, Belgium; February 2012</td>
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<td>Water with detritus of a forest pool</td>
<td>Natural reserve Mydlovarske manzury, Czech Republic; May 2014</td>
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*ANC, Designations used by the Laboratory of Bacterial Genetics, Prague, Czech Republic; UG, University of Ghent, Belgium.
GPS coordinates, 50.1765697 N 14.9253897 E.
Acinetobacter gandensis sp. nov.

Fig. 1. Estimated phylogenetic relationships of members of the genus *Acinetobacter* shown by a neighbour-joining tree based on the 16S rRNA gene sequences [between base position 174 and 1422 according to the *Escherichia coli* numbering (Brosius et al., 1978)] of the six strains of *Acinetobacter gandensis* sp. nov. and strains of species of the genus *Acinetobacter*. The sequence of *Moraxella lacunata* LMG 5301\(^T\) (NCBI accession no. D64049) was used as the outgroup. Bar, 2 substitution per 100 nt. A similar tree topology was obtained using the Maximum-likelihood method (not shown).
accession numbers KC631629.1, KJ124834.1 and EU477150.2, respectively).

Based on 16S rRNA gene and rpoB comparative analyses, the six strains do not belong to any of the known species with validly published names or to genomic species with provisional designation (DNA groups 6, 15TU, 13–17BJ), but they are related to each other below the species level.

For DNA–DNA hybridization, genomic DNA was isolated according to a modification (Cleenwerck et al., 2002) of the procedure of Wilson (1987). Based on both similarity values for the 16S rRNA gene and the rpoB sequences and on cluster analysis results, A. schindleri LMG 19576T was selected to carry out DNA–DNA hybridization with strains UG 60467T and UG 60716. DNA–DNA hybridization was determined at the Laboratory for Microbiology, Ghent, Belgium (LMG), as described previously (Goris et al., 1998; Cleenwerck et al., 2002). Strains UG 60467T and UG 60716 showed a DNA–DNA relatedness value of 84 %, which is above the threshold of 70 % generally accepted for species delineation (Wayne et al., 1987), and both showed a DNA–DNA relatedness value clearly below this with A. schindleri LMG 19576T, i.e. 17 % and 20 %, respectively.

![Fig. 2. Rooted neighbour-joining dendrogram based on the rpoB partial nucleotide sequences (861 bp) of the six strains of Acinetobacter gandensis sp. nov. and strains of species of the genus Acinetobacter.](image-url)
The DNA G+C content of strain UG 60467T was determined by the LMG by means of HPLC (Mesbah et al., 1989), taking the mean of three independent analyses of the same DNA sample. The DNA G+C content of 39.6 mol% falls within the range for species of the genus Acinetobacter, i.e. 36.6–47.0 mol% (Álvarez-Pe´rez et al., 2013; Juni, 2005; Kim et al., 2008; Li et al., 2013, 2014; Vaz-Moreira et al., 2011).

For fatty acid analysis, cells were grown for 24 h at 28 °C under aerobic conditions on tryptic soy agar culture plates (Becton Dickinson). Inoculation and harvesting of the cells, and the extraction and analysis were performed according to the recommendations of the Microbial Identification System (MIDI), except that cells were harvested from the whole plate to obtain a sufficient concentration of fatty acids in the extract. The whole-cell fatty acid composition was determined using gas chromatography. The predominant fatty acids for strain UG 60467T were C12 : 0 3-OH (7.4 %), C12 : 0 (10.5 %), C16 : 0 (14.0 %), C18 : 1ω9c (19.9 %) and summed feature 3 (containing the fatty acids C16 : 1ω7c and/or iso-C15 : 0 2-OH that cannot be differentiated by the MIDI system) (32.9 %).

The nutritional and physiological tests were carried out as described by Nemec et al. (2010, 2011) and Krizova et al. (2014). The in-house assimilation tests were performed in fluid mineral medium, supplemented with 0.1 % (w/v) carbon source. Temperature growth tests were performed in Brain-Heart Infusion broth (Oxoid), using a thermostatically controlled water bath. Phenotypic analysis was also carried

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Table 2. Phenotypic characteristics of Acinetobacter gandensis sp. nov. and phenotypically most similar species of the genus Acinetobacter

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Fig. 3. a) Dendrogram based on the whole-cell MALDI-TOF mass spectra of six strains of *Acinetobacter gandensis* sp. nov. and the type/reference strains of other species of the genus *Acinetobacter*. The dendrogram was constructed using the correlation distance measure with the average linkage algorithm (UPGMA). b) Combined spectra of the strains of *A. gandensis* sp. nov. with the peaks present in all six strains indicated by molecular mass.
out using the API 20NE system (bioMérieux), according to
the manufacturer’s instructions. The cultivation temperature
was 30°C unless indicated otherwise, and all tests were
performed at least twice independently and repeated when
inconsistent results were obtained.

The six novel strains were phenotypically compared to the
strains included in the Acinetobacter collection of the
Laboratory of Bacterial Genetics, Prague, Czech Republic,
which encompasses all species with validly published
names, genomic species, and a number of tentative species.
Overall, the novel strains were capable of utilizing a rather
limited number of carbon sources and thus could be easily
differentiated from biochemically more active species of
the genus Acinetobacter. Table 2 shows the phenotypes of
the six novel strains and of the phenotypically most similar
species, i.e. those which differed from the novel strains in
less than five of the traits listed in the table, taking into
consideration only the traits that were either positive or
negative in all strains of a species. The novel strains were
unambiguously differentiated from some of these species
(Acinetobacter indicus, A. johnsonii, Acinetobacter kloekii,
Acinetobacter parvus, Acinetobacter fijnbergiae, Acinetobacter
ursingii and genomic species 15TU) by at least two
properties. The remaining species could be separated from
the novel strains by one clear trait and/or combination of
several features found in most strains of a given species.
Amongst these, the species most similar to the novel strains
were Acinetobacter junii, which can be differentiated from
the novel strains by its growth at 41°C and its assimilation of L-
histidine and L-arginine; A. bouvetii, which assimilates L-
histidine; A. schindleri, which grows at 41°C and assimilates
D-malate; and Acinetobacter harbinensis, which does not
grow at 37°C. Acinetobacter towneri could not be differ-
entiated reliably from the novel strains by any property
except for its weak growth at 41°C.

For matrix assisted laser desorption ionization-time of flight
(MALDI-TOF) MS analysis, a standard protocol based on
extraction with acetonitrile/formic acid/water and alpha-
cyano-4-hydroxycinnamic acid used as matrix was adapted
from Freiwald & Sauer (2009), as described previously
(Krizova et al., 2014). MALDI-TOF mass spectra measure-
ments were carried out using an Ultraflex III instrument
(Bruker Daltonics), operated in linear positive mode under
control of the FlexControl 3.0 software. Mass spectra were
processed using the Flex Analysis (version 3.4; Bruker
Daltonics) and BioTyper software (version 3.1; Bruker
Daltonics). The parameters of methods used for calibration,
acquisition and evaluation of the obtained mass spectra were
set as described (Krizova et al., 2014).

The six novel strains, together with the type strains of all
species of the genus Acinetobacter with validly published
names and reference strains of the other species of the
genus Acinetobacter, were subjected to MALDI-TOF MS
whole-cell profiling analysis (Fig. 3). All spectral patterns of
the novel strains showed a high degree of mutual similarity,
with a total of 23 consensus peaks detected (Fig. 3b), which
is also reflected by their clustering in a dendrogram (Fig.
3a). All spectral patterns of the novel strains were clearly
different from those of the other species of the genus
Acinetobacter.

Based on data obtained in this study, the six novel strains
represent a novel species of the genus Acinetobacter, for
which the name Acinetobacter gandensis sp. nov. is proposed.

**Description of Acinetobacter gandensis sp. nov.**

Acinetobacter gandensis (gand.en’sis. N.L. masc. adj. gand-
ensis pertaining to Ghent, Belgium, named as such because
the first three strains were isolated in the vicinity of Ghent,
i.e. at the Faculty of Veterinary Medicine in Merelbeke).

The species description is based on the characterization of
divation. Acid is not produced from D-glucose, gelatin
is not hydrolysed, and sheep erythrocytes are not lysed.
Acetate, benzoate, ethanol and DL-lactate are utilized as the
sole sources of carbon with growth visible within four (mostly
two) days of incubation. No growth occurs on trans-
aconitate, adipate, β-alanine, 4-aminoobutyrate, L-arginine,
L-aspartate, azelate, citraconate, gentisate, L-histidine, 4-hydroxybenzoate,
L-leucine, D-glucuonate, D-glucose, levulinate, histamine, D-
malate, L-ornithine, phenylacetate, L-phenylalanine, L-
tartrate, putrescine, tricarballylate, trigonelline or tryptamine.
Utilization of 2,3-butandiol, citrate (Simmons), L-glutamate,
glutarate and malonate is variable. Growth on L-
arabinose or D-ribose is either negative (mostly) or difficult
to interpret.

The type strain is UG 60467T (=ANC 4275T =LMG
27960T =DSM 28097T), isolated in February 2012 from
dung of a hospitalized horse, with an illness not associated
with the isolate. The type strain grows on citrate (Simmons),
L-glutamate and glutarate, but not on 2,3-butandiol or
malonate. The predominant fatty acids of the type strain are
C12:0 3-OH, C12:0 3-OH, C16:0 and summed feature 3
(C16:1t07c and/or iso-C15:0 2-OH). The DNA G+C content of
the type strain is 39.6 mol%.

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Addendum: After acceptance of this manuscript we became aware of
the deposit of sequence data by G. Wilharm and L. Jerzak referring to
two Acinetobacter isolates collected from white stork nesting in

http://ijs.sgmjournals.org
Poland. Based on the rpoB partial sequence data deposited (KJ135349; KJ135350) in all likelihood these isolates belong to Acinetobacter gandensis sp. nov. This work was supported by internal funding by the Department of Pathology, Bacteriology and Avian Diseases, Ghent University, and by a grant from the Czech Science Foundation (grant no. 13-26693S).

References


