Acinetobacter dijkshoorniae sp. nov., a member of the Acinetobacter calcoaceticus–Acinetobacter baumannii complex mainly recovered from clinical samples in different countries

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The recent advances in bacterial species identification methods have led to the rapid taxonomic diversification of the genus Acinetobacter. In the present study, phenotypic and molecular methods have been used to determine the taxonomic position of a group of 12 genotypically distinct strains belonging to the Acinetobacter calcoaceticus–Acinetobacter baumannii (ACB) complex, initially described by Gerner-Smidt and Tjernberg in 1993, that are closely related to Acinetobacter pittii. Strains characterized in this study originated mostly from human samples obtained in different countries over a period of 15 years. rpoB gene sequences and multilocus sequence typing were used for comparisons against 94 strains representing all species included in the ACB complex. Cluster analysis based on such sequences showed that all 12 strains grouped together in a distinct clade closest to Acinetobacter pittii that was supported by bootstrap values of 99%. Values of average nucleotide identity based on BLAST between the genome sequence of strain JVAP01T (NCBI accession no. LJPG00000000) and those of other species from the ACB complex were always <91.2%, supporting the species status of the group. In addition, the metabolic characteristics of the group matched those of the ACB complex and the analysis of their protein signatures by matrix-assisted laser desorption ionization time-of-

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Abbreviations: ACB complex, Acinetobacter calcoaceticus–Acinetobacter baumannii complex; ANIb, average nucleotide identity based on BLAST; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MLSA, multilocus sequence analysis; MLST, multilocus sequence typing; PFGE, pulse-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences determined in this study are: KU167837, KU167840, KU167842, KU167844, KU167846, KU167848–KU167851, KU167853–KU167858, KU167860–KU167865, KU167867–KU167869, KU167871, KU167873–KU167885, KU167888 and KU726606 for the partial sequence of the rpoB genes and KX027435 for the complete sequence of the 16S rRNA gene of Acinetobacter dijkshoorniae sp. nov. strain JVAP01T. The partial sequences of the seven genes used for MLSA are available from the PubMLST website (http://pubmlst.org/abaumannii/) under the sequence type codes listed in Table S1.

Six supplementary tables and two supplementary figures are available with the online Supplementary Material.
The genus *Acinetobacter* comprises, at the time of writing, 49 validly named species (http://www.bacterio.cict.fr/a/acinetobacter.html), including two recently described in this journal (Li *et al.*, 2015; Poppel *et al.*, 2015). In addition, there are a number of putative novel species without validly published names, several of which were identified as a result of recent advances in DNA-based methods. Since 2014, 17 new *Acinetobacter* species have been validly described, notably *Acinetobacter seifertii*, a novel human pathogen included within the ‘*Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex’ (Du *et al.*, 2016; Feng *et al.*, 2014a, b; Kim *et al.*, 2014b; Krizova *et al.*, 2014, 2015a, b; Li *et al.*, 2014a, b, 2015; Nemec *et al.*, 2015, 2016; Poppel *et al.*, 2015; Smet *et al.*, 2014). The ACB complex includes five different species, the environmental *A. calcoaceticus* and the human pathogens *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *A. seifertii* (Nemec *et al.*, 2015). Members of this complex are difficult to distinguish phenotypically and are also closely related to each other based on DNA–DNA hybridization, to the point that accurate differentiation of the four clinically relevant members of the group can only be accomplished by means of molecular methods (Gundi *et al.*, 2009; Sousa *et al.*, 2014).

The introduction of molecular typing methods in the routine characterization of *Acinetobacter* clinical specimens recently allowed us to identify a group of strains highly related to other members of the ACB complex that nevertheless seemed to constitute a novel *Acinetobacter* species (Espinal *et al.*, 2015). In the present work we have used phenotypic and molecular methods relevant to the genus *Acinetobacter* to assess the taxonomic position of these strains and support the designation of another member of the ACB complex with putative clinical relevance.

The strains characterized in this work were mainly obtained from clinical samples of patients in different geographical locations over a period of 15 years and the results shown below clearly support the taxonomic distinctness of the group at the species level. We propose the name *Acinetobacter dijkshoorniae* sp. nov. to accommodate these strains, which will be used hereafter.

Fifteen *A. dijkshoorniae* sp. nov. isolates were initially included in the study. Their genetic relatedness was evaluated by *Apa* macrorestriction of genomic DNA and subsequent analysis of the pulse-field gel electrophoresis (PFGE) fingerprints (Seifert *et al.*, 2005). As shown in Fig. 1, 12 genetically distinct fingerprints were identified, with three pairs of isolates presenting identical PFGE patterns. For the purpose of the present study, 12 genetically unique strains were selected and subsequently studied following a polyphasic approach using both genomic and phenotypic techniques.

The partial *rpoB* gene sequences (zones 1 and 2) of all 12 *A. dijkshoorniae* sp. nov. isolates (La Scola *et al.*, 2006) were compared to those of 93 reference strains belonging to other members of the ACB complex, according to Nemec *et al.* (2011). Data on the origin and characteristics of the strains used for sequence analysis are shown in Table S1, available in the online Supplementary Material. Cluster analysis was performed using the *MEGA 5.1* software (Tamura *et al.*, 2011). The program *MUSCLE* was used for sequence alignment (Edgar, 2004) and phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood methods, with genetic distances computed by Kimura’s two-parameter model (Kimura, 1980), but also using the maximum-parsimony method, all three with bootstrap values based on 1000 replicates.

Results of the *rpoB*-gene-based sequence analysis confirmed the genetic distinctness of all selected *A. dijkshoorniae* sp. nov. isolates and showed their grouping together in a tight monophyletic group within the ACB complex that was closest to *A. pittii*. Representative strains from all other species of the ACB complex also formed respective clusters that were supported by high bootstrap values, as shown in Fig. 2(a). The intraspecies *rpoB* gene sequence similarity values of *A. dijkshoorniae* sp. nov. strains ranged between 98.61 and 100 %, while the interspecies similarity values between *A. dijkshoorniae* sp. nov. and the other ACB complex strains ranged from 91.29 % (*A. seifertii* ANC 4233) to 98.03 % (*A. pittii* JVAP02) (Table S2). Intraspecies and interspecies *rpoB* gene sequence similarity values for all other taxa were also in good agreement with similarity values previously published (Nemec *et al.*, 2015).

The distinct grouping of all strains in well-defined phylogenetic taxa was further evaluated using multilocus sequence analysis (MLSA) of the concatenated partial sequences of all housekeeping genes used for multilocus sequence typing (MLST) according to the Pasteur scheme (Diancourt *et al.*, 2010).

The partial sequences of the *cpr60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB* genes for all strains were either obtained by PCR amplification and subsequent DNA sequencing, as described by Diancourt *et al.* (2010), or retrieved from nucleotide sequence repositories whenever available (Table S1). New allele sequences as well as unique allele combinations were submitted to the *Acinetobacter* MLST database (http://pubmlst.org/abauumannii/) and were used to assign novel sequence types (Table S1).

As shown in Fig. 2(b), MLSA was in very good agreement with results from *rpoB* gene sequence comparisons, with all
A. dijkshoorniae sp. nov. strains forming again a separate monophyletic group that was closer to A. pittii. MLSA interspecies similarity values for A. dijkshoorniae sp. nov. were 98.82–100 % and the MLSA interspecies similarity values between A. dijkshoorniae sp. nov. and all other ACB complex species ranged from 91.4 % (A. baumannii NIPH 1734) to 98.89 % (A. pittii JV-2628), suggesting a high degree of genetic relatedness between A. dijkshoorniae sp. nov. and A. pittii (Table S3).

Cluster analysis based on the independent partial sequences of each of the seven MLST genes showed little horizontal transfer between the other species of the ACB complex and strains of A. dijkshoorniae sp. nov., with only the fusA allele of strain LUH 08258, the recA allele of strain RUH 14531 and the rpoB allele of strain LUH 10297 clustering together with the respective sequences of A. pittii (Fig. S1). Limited horizontal transfer between closely related species of the ACB complex has been previously reported (Nemec et al., 2015), thus reinforcing the use of MLSA instead of that of that of MLSA-based sequence analysis, was 96.64 %, further supporting the distinction of this novel species from other members of the ACB complex (Table S5).

Results from the ANIb analyses (Table S4) proved the species status of A. dijkshoorniae sp. nov. since identity values between the JVAP01T genome and other ACB complex species genomes were below the proposed species demarcation level of 95 % (Kim et al., 2014a), ranging from 86.74 % (A. nosocomialis ATCC 17903) to 93.13 % (A. pittii RUH 2206T). Interestingly, the ANIb value of the JVAP01T genome against that of strain SCOPE 172, which clustered together with A. dijkshoorniae sp. nov. strains in both the rpoB gene- and MLSA-based sequence analysis, was 96.64 %, further supporting the distinctness of this novel species from other members of the ACB complex (Table S5).

The genome sequence of A. dijkshoorniae sp. nov. strain JVAP01T contains three complete and identical sequences of the 16S rRNA gene (1526 bp) and two partial sequences, and that of strain SCOPE 271 contains six complete and identical 16S rRNA gene sequences. The nearly complete 16S rRNA gene sequences (positions 48–1450) from all other A. dijkshoorniae sp. nov. strains included in the study were obtained by Sanger
sequencing. The intraspecies 16S rRNA gene sequence similarity values of A. dijkshoorniae sp. nov. strains ranged between 98.36 and 100 %, while the interspecies similarity values between A. dijkshoorniae sp. nov. and the other ACB complex strains ranged from 96.15 % (A. baumannii) to 99.57 % (Acinetobacter sp. 'Between 1 and 3'). Similarity values to A. calcoaceticus ranged between 98.57 and 99.22 % and to A. pittii between 98.29 and 99.14 %. Cluster analysis also grouped A. dijkshoorniae sp. nov. strains closer to Acinetobacter sp. 'Between 1 and 3' than to A. pittii, although all A. dijkshoorniae sp. nov. strains formed a single monophyletic group clearly separated from the other species (Fig. S2).

Likewise, the four different 16S rRNA gene sequences from A. seifertii NIPH 973 could not be clearly allocated as a unique taxon. This result supports previous studies showing that differentiation of species within the ACB complex based on 16S rRNA gene sequence analysis is of little value, the rpoB gene being much more reliable for differentiation of this group of highly related species (Gundi et al., 2009; Nemec et al., 2015).

To further evaluate the phenotypic relationships among strains clustering together as A. dijkshoorniae sp. nov., bacterial extracts were obtained using the ethanol/formic acid extraction method as previously described (Espinal et al., 2012) and analysed by matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS). Spectra profiles were acquired with a Microflex LT (Bruker Daltonik) benchtop instrument in linear positive mode at a laser frequency of 20 Hz. The resulting raw spectra were loaded into the ClinProTools software (version 2.2; Bruker Daltonics), grouped according to each bacterial species within the ACB complex, and used to calculate the average spectra for each species. Mass to charge values (m/z) from the average spectra of each species were extracted and we identified three peaks at 4429, 5788 and 8856 m/z values that were present in all A. dijkshoorniae sp. nov. strains but absent in all the spectra from other ACB species (Fig. 3). The presence/absence of such A. dijkshoorniae sp. nov. specific peaks could potentially be used for MALDI-TOF MS direct discrimination of strains of A. dijkshoorniae sp. nov.

Carbon utilization assays were performed to assess the phenotypic traits of the different species as previously described (Bouvet & Grimont, 1987; Krizova et al., 2014; Nemec et al., 2010, 2011) with minor modifications. Briefly, inocula were prepared by growing strains on tryptic soy agar (TSA; Oxoid) at 30 °C for 24 h and subcultured once under the same conditions before being used. Cells were removed with a cotton swab and suspended in a carbonless mineral broth medium until a transmittance of 90 % (turbidimeter;
Overall, the metabolic traits of the *Acinetobacter dijkshoorniae* sp. nov. strains were in good agreement with those shown by Nemec et al. (2015) and the results obtained in the present study.

**Description of Acinetobacter dijkshoorniae** sp. nov.

*Acinetobacter* dijkshoorniae (dijkshoorn'i.ae. N.L. gen. fem. n. dijkshoorniae of Dijkshoorn, named after Lenie Dijkshoorn, a Dutch microbiologist, for her life-long dedication to the taxonomy of the genus *Acinetobacter*).

Phenotypic characteristics correspond to those of the genus, i.e. Gram-stain-negative, strictly aerobic, oxidase-negative, catalase-positive cocccobacilli, incapable of swimming motility, capable of growing in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen, and incapable of dissimilative denitrification. Colonies on TSA after 24 h of incubation at 30°C are 1.0–2.0 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs in brain heart infusion broth (BD Difco) at temperatures ranging from 25 to 44°C in 1 day. All strains produce acid from D-glucose and fail to hydrolyse gelatin. Haemolysis of erythrocytes on sheep blood agar medium is not observed. All strains are able to use acetate, trans-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, azelate, benzoate, 2,3-butanediol, citrate, gentisate, L-glutamate, glutarate, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, D-malate, malonate, L-phenylalanine, putrescine, tricarballylate and L-tyrosine as a sole carbon source. None of the tested strains are able to use citraconate, D-glucuronate, D-glucose or histamine as a carbon source and ammonia as the sole source of nitrogen, and incapable of dissimilative denitrification. Colonies on TSA after 24 h of incubation at 30°C are 1.0–2.0 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs in brain heart infusion broth (BD Difco) at temperatures ranging from 25 to 44°C in 1 day. All strains produce acid from D-glucose and fail to hydrolyse gelatin. Haemolysis of erythrocytes on sheep blood agar medium is not observed. All strains are able to use acetate, trans-aconitate, adipate, β-alanine, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, azelate, benzoate, 2,3-butanediol, citrate, gentisate, L-glutamate, glutarate, L-histidine, 4-hydroxybenzoate, DL-lactate, L-leucine, D-malate, malonate, L-phenylalanine, putrescine, tricarballylate and L-tyrosine as a sole carbon source. None of the tested strains are able to use citraconate, D-glucuronate, D-glucose or histamine for growth. Some strains are able to grow on ethanol, levulinate, L-ornithine, phenylacetate, D-ribose, L-tartrate, trigonelline and triptamine.

The type strain is JVAP01\(^T\) (=CECT 9134\(^T\)=LMG 29605\(^T\)), isolated from a urine sample of a human patient in Turkey in August 2009 (Espinal et al., 2015). In addition to the
above-mentioned carbon sources, the type strain is also able to utilize L-ornithine, phenylacetate, D-ribose and trigonelline as a carbon source. Growth on tryptamine is also observed although weakly. No growth is observed with citraconate, ethanol, D-glucorants, D-glucose, levulinate or L-tartrate. MALDI-TOF MS analysis of JVAP01 showed the presence of the A. dijkshoorniae sp. nov. specific peaks centered at 4429, 5788 and 8856 m/z values. The whole genome sequence of the type strain is available from NCBI under accession number LJPG00000000 (size 3.85 Mb, number of contigs 92, DNA G+C content 38.8 mol%). The GenBank/EMLB/DDBJ accession numbers for the partial sequence of the rpoB gene and the complete 16S RNA gene sequence of strain JVAP01 are KJ600793 and KX027435, respectively.

Acknowledgements

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References


Table 1. Metabolic characteristics of A. dijkshoorniae sp. nov. and other species of the ACB complex

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