Acinetobacter defluvii sp. nov., recovered from hospital sewage

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Abstract
A Gram-stain-negative, non-motile Acinetobacter strain, WCHA30T, was isolated from hospital sewage in West China Hospital of Sichuan University in Chengdu, south-western China. Strain WCHA30T was a non-spore-forming, catalase-positive, oxidase-negative, strictly aerobic coccobacillus. The DNA G+C content was 38 mol%.

The genus Acinetobacter was first proposed by Brisou and Prevot [1]. Common characteristics of Acinetobacter members are that they are are Gram-negative, strictly aerobic, catalase-positive, oxidase-negative and non-motile except twitching or swarming. Members of the genus Acinetobacter are distributed widely in nature and some Acinetobacter species have been recovered from clinical specimens such as blood, intravascular catheters, skin, ear, eye and sputum [2–5]. However, many species of the genus Acinetobacter have been isolated from various environmental sources such as wetlands, river water, activated sludge and abandoned ore [6–12]. As of January 2017, the formal classification of the genus Acinetobacter included 53 validly published names including two pairs of synonyms (www.szu.cz/anemec/Classification.pdf). In addition, there are seven Acinetobacter species with tentative designations (www.szu.cz/anemec/Classification.pdf). In the present study, strain WCHA30T was recovered from hospital sewage of West China Hospital, Sichuan University, Chengdu, south-western China. The strain is distinct from all hitherto known genomic species and therefore may represent a novel species of the genus Acinetobacter.

Hospital sewage was collected from the influx mainstream of the wastewater treatment plant in West China Hospital in November 2015. The sewage (1 ml) was mixed with 100 ml of nutrient broth (Oxoid) in a 500 ml flask and was incubated overnight at 30 °C with shaking. The culture suspension was diluted to 0.5 McFarland standard and was then further diluted to 1:100 using saline. A 100 μl aliquot was plated onto an Acinetobacter chromogenic agar plate (CHROMagar) containing 32 μg meropenem ml–1 and 32 μg linezolid ml–1. Linezolid was added to inhibit the growth of Gram-positive bacteria, while meropenem was added to screen carbapenem-resistant bacterial isolates. Plates were then incubated at 30 °C overnight. Strain WCHA30T was recovered and was subsequently cryopreserved at −80 °C in Luria–Bertani (LB) broth as 30 % (v/v) glycerol suspensions. Strain WCHA30T was resistant to meropenem (MIC, 16 μg ml–1) as determined using the microdilution broth method following recommendations of the Clinical Laboratory Standards Institute [13] and was found to carry the carbapenemase gene blaNDM-1, by PCR and Sanger sequencing as described previously [14].

Boiled lysates were used as the PCR template and PCR amplicons were sequenced in both directions at the Beijing Genomics Institute (Chongqing, China). The 16S rRNA gene of strain WCHA30T was amplified by PCR using the universal primers 27F and 1492R [15]. Whole genome sequences are available for 49 of the 51 Acinetobacter species type strains with validly published names and for all of the reference strains of the seven Acinetobacter species with tentative species designations. The 16S rRNA gene sequences of the type or reference strain of these 56 Acinetobacter species were retrieved from their whole genome sequences. There are no whole genome sequences available for Acinetobacter guangdongensis and Acinetobacter populi and their...
1456 bp partial 16S rRNA gene sequences were retrieved from GenBank. A phylogenetic tree of the genus Acinetobacter (Fig. 1) based on the 16S RNA gene sequences was generated using MEGA 7.0 [16] with the neighbour-joining method [17] and partial deletion of gaps and site coverage cut-off of 95%. The 16S rRNA gene sequence of Moraxella lacunata ATCC 17967T (GenBank accession no. AF005160) was used as the outgroup. Compared to those of the type or reference strain of each Acinetobacter species, the obtained 1456 bp 16S rRNA gene sequence of strain WCHA30T (GenBank accession no. KY435933) was most similar (98.1%) to that of Acinetobacter bereziniiae CIP 70.12T. The phylogenetic tree also suggested that strain WCHA30T was most closely related to A. bereziniiae CIP 70.12T and Acinetobacter guillouiae CIP 63.46T (Fig. 1).

Genomic DNA of strain WCHA30T was prepared using a QIAamp DNA Mini Kit (Qiagen) and was subjected to whole genome sequencing using the HiSeq 2500 Sequencing platform (Illumina) following the manufacturer’s protocol, at Novogene (Beijing, China). The program SPAdes [18] was used to assemble reads into contigs. Annotation of the genomic sequence was carried out using Prokka [19]. A total of 5 106 848 clean reads and 1.53 Gb clean bases were generated for strain WCHA30T, which were assembled into 74 contigs ≥1000 bp in length (N50, the shortest sequence length at 50% of the genome, 139 774 bp) with a G+C content of 38.0 mol%. The draft whole genome sequence of strain WCHA30T has been deposited in GenBank under accession number MAUF0000000.

Similarity calculations and cluster analysis of the rpoB (RNA polymerase – subunit) and gyrB (DNA gyrase subunit B) gene sequences among Acinetobacter species were carried out using partial sequences of rpoB (861 bp) and gyrB (891 bp), corresponding to nucleotide positions 2915–3775 and 303–1193 of the coding region of these genes in Acinetobacter baumannii CIP 70.34T (GenBank accession no. APRG000000000), respectively. The rpoB and gyrB gene sequences of the type or reference strain of each species within the genus Acinetobacter were retrieved from whole genome sequences except for A. guangdongensis and A. populi, for which their sequences deposited in GenBank were used. Neighbour-joining trees for rpoB (Fig. S1, available in the online Supplementary Material) and gyrB (Fig. S2) were generated as described for the 16S rRNA gene (see above). The similarity of the rpoB gene sequence between strain WCHA30T (GenBank accession no. KY435935) and strains of other Acinetobacter species ranged from 78.4% (Acinetobacter qingfengensis ANC 4671T) to 87.5% (Acinetobacter gandensis ANC 4275T). The similarity of the gyrB gene sequence between strain WCHA30T (GenBank accession no. KY435934) and strains of other Acinetobacter species ranged from 76.1% (Acinetobacter apis ANC5114T) to 83.4% (A. guillouiae CIP 63.46T). Phylogenetic analysis based on 16S rRNA, rpoB and gyrB gene sequences suggested that strain WCHA30T represents a novel species within the genus Acinetobacter.

The partial sequences of seven housekeeping genes (i.e. cpn60, fusiA, gllA, pyrG, recA, rplB and rpoB) used for A. baumannii multilocus sequence typing (MLST) in the Pasteur scheme (pubmlst.org/abaumannii) were identified from the whole genome sequence of the type or reference strain of each Acinetobacter species listed in Table S1, using the online tool MLST 1.8 [20]. These were aligned using Clustal Omega [21] and then concatenated for each strain. The phylogenetic tree was inferred from the concatenated sequences by MEGA 7.0 [16] using the neighbour-joining method with Kimura’s two-parameter model and 1000 bootstrap replicates as the nucleotide substitution model and method to test phylogeny, respectively. The MLST-based tree (Fig. S3) also shows that strain WCHA30T is distinct from other Acinetobacter species.

An average nucleotide identity (ANI) value of ≥95% has been proposed to define a species within the genus Acinetobacter based on a limited database [22]. ANI based on BLAST was calculated for the genome sequence of strain WCHA30T and those of a type or reference strain of each of the 56 Acinetobacter species with the genome sequence available in GenBank (Table S1) using the JSpecies web program (imedea.uib-csic.es/jspecies/) with default settings [23]. ANI values of the genome sequence of strain WCHA30T and all other type or reference Acinetobacter genomes including those of A. bereziniiae CIP 70.12T and A. guillouiae CIP 63.46T were <80%, with the highest value being 79.5% to that of A. bereziniiae CIP 70.12T (GenBank accession no. APQG01000000). Although the ≥95% cutoff or any other cutoffs to define an Acinetobacter species require rigorous verification, the <80% ANI values were far below any proposed cutoff to define a bacterial species, suggesting that strain WCHA30T is very likely to represent a novel Acinetobacter species.

DNA–DNA hybridization was determined using a Beckman DU800 spectrophotometer following the initial renaturation rate method [24] at the China Center of Industrial Culture Collection. The level of DNA–DNA relatedness between strain WCHA30T and A. bereziniiae CIP 70.12T was only 47.4%, which was lower than the 70% cutoff to define a bacterial species, indicating that the two strains belonged to different species. In silico DNA–DNA hybridization between strain WCHA30T and the type or reference strain of 56 Acinetobacter species with the genome sequence available in GenBank (Table S1) was performed using GGDC (formula 2) [25]. The in silico DNA–DNA relatedness between strain WCHA30T and any of the 56 Acinetobacter species ranged from 20.0 to 27.9%, much lower than the 70% cutoff, which provides further support that strain WCHA30T belongs to a novel Acinetobacter species.

The motility of strain WCHA30T was examined using light microscopy and cell morphology was observed using transmission electron microscopy (H-7650; Hitachi), with cells grown at 30°C on LB agar for 24 h. Growth at various temperatures (20, 25, 30, 32, 35, 37, 41 and 44°C) was determined in test tubes containing 3 ml tryptic soy broth (TSB;
**Fig. 1.** Neighbour-joining tree based on the partial 16S rRNA gene nucleotide sequences of strain WCHA30\(^T\), the type strains of Acinetobacter species with validly published names and reference strains of several tentative species designations. M. lacunata ATCC 17967\(^T\) (=NBRC 102154\(^T\)) was used as an outgroup. Bootstrap values >50% (based on 1000 resamplings) are shown. Bar, 0.005 substitutions per nucleotide position.
Hopebio) after incubation for 2 days in a thermostatically controlled water bath as described previously [5]. Growth at different NaCl concentrations (0–10 %, w/v, in increments of 1.0 %) was tested in TSB for 2 days at 30 °C. The pH range (pH 4–10, at intervals of 1 pH unit, modulated by adding HCl or NaOH) for growth was investigated in TSB after incubation for 2 days at 30 °C. Growth in an anaerobic bag (bioMérieux) was checked on LB agar plates at 30 °C for 7 days. Acid production from glucose under aerobic conditions and gelatin hydrolysis were examined according to methods described previously [5]. The cultivation temperature was 30 °C unless indicated otherwise. The results of the above tests are given in the species description.

Phenotypic analysis was carried out using the genus-targeted set of standardized tests as described previously [5, 26]. The results of the tests for strain WCHA30T are shown together with those obtained from the literature of all Acinetobacter species with validly published names in Table S2. Strain WCHA30T differed from each of the known species in four to 22 of the 40 characteristics studied. Furthermore, the combination of its ability to assimilate β-alanine but not l-glutamate distinguished strain WCHA30T from all known Acinetobacter species, as shown in Table S2.

Genotypic and phenotypic characteristics and the genome sequence of strain WCHA30T support the suggestion that the strain should be considered to represent a novel species of the genus Acinetobacter, for which the name Acinetobacter defluvii sp. nov. is proposed. The type strain is WCHA30T. Of note, we obtained only one strain for the proposed A. defluvii sp. nov., which is a limitation of the study as, ideally, multiple strains are required to describe a novel species.

DESCRIPTION OF ACINETOBACTER DEFLUVII SP. NOV.

Acinetobacter defluvii (de.flu’vi.i. L. gen. n. defluvii of sewage).

Cells are Gram-stain-negative, non-motile, non-spor-forming, catalase-positive, oxidase-negative, strictly aerobic coccobacilli (0.6–0.9×0.7–1.0 μm). Cells grow on media such as tryptic soy agar (TSA; Oxoid), LB agar, brain heart infusion agar and Müller-Hinton agar (all from Hopebio). Colonies on TSA are light yellow, circular, smooth, semi-transparent, slightly convex with entire margins, and approximately 0.8–1.2 mm in diameter after 24 h of incubation at 30 °C. Growth occurs in 0–3 % (w/v) NaCl and at 20–37 °C, but not at 41–44 °C within 2 days. Cells grow at pH 5–10. Acid is not produced from D-glucose and gelatin is not hydrolysed but Tween 40 is hydrolysed. Haemolysis is observed on agar media supplemented with sheep erythrocytes. Acetate, β-alanine, 4-aminobutyrate, benzoate, ethanol, Dl-lactate, phenylacetalde and trigonelline can be utilized as sole sources of carbon, with growth becoming visible in 6 (generally 2) days of culture at 30 °C. No growth occurs on trans-aconitate, adipate, l-arabinose, l-aspartate, azelate, 2,3-butanediol, citraconate, citrate (Simmons), gentisate, D-glucosone, D-glucose, l-glutamate, glutarate, histamine, l-histidine, 4-hydroxybenzoate, l-leucine, levulinate, D-malate, malonate, l-ornithine, l-phenylalanine, putrescine, D-ribose, l-tartrate, tricarboxylate or tryptamine.

The type strain is WCHA30T (=CCTCC AB 2016203T =GDMCC 1.1101T=KCTC 52503T), isolated from hospital sewage in West China Hospital of Sichuan University in Chengdu, China.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


