Acinetobacter harbinensis sp. nov., isolated from river water

Weiguang Li,1,2 Duoying Zhang,1,3 Xiaofei Huang1 and Wen Qin1

1School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150090, PR China
2State Key Laboratory of Urban Water Resource and Environment, Harbin 150090, PR China
3Department of Civil and Environmental Engineering, Aalto University, P.O. Box 15200, FI-00076 Aalto, Finland

A bacterial strain, HITLi 7T, with nitrifying ability was isolated from the surface water of the Songhua River in China. Cells were Gram-stain-negative, strictly aerobic, oxidase-negative, non-motile cocobacilli, capable of growth in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen. The cells did not grow at 37 °C, but did grow at 2 °C. The DNA G+C content was 45.5 mol%. Results of 16S rRNA gene sequence analysis indicated a close relationship between this isolate and Acinetobacter lwofii (98.4 % similarity for strain DSM 2403T). rpoB and gyrB gene sequences did not show significant similarity with those from other species of the genus Acinetobacter. Predominant cellular fatty acids were 9-octadecenoic acid (C18:1ω9c) and summed feature 4 (iso-C15:0 2-OH and/or C16:1ω7c). Acid was not produced from D-glucose, and gelatin was not hydrolysed by the isolate. Genotypic, phenotypic and chemotaxonomic data from this study indicate that the isolate should be classified as a representative of a novel species of the genus Acinetobacter. The name Acinetobacter harbinensis sp. nov. is proposed for the novel species, with HITLi 7T (=CGMCC 1.12528T=KCTC 32411T) as the type strain.

The genus Acinetobacter was first proposed by Brisou & Prévot (1954) in the phylum Proteobacteria, family Moraxellaceae. The type species is Acinetobacter calcoaceticus. At the time of writing, 31 species with validly published names (http://www.bacterio.net/a/acinetobacter.html; list of prokaryotic names with standing in nomenclature) and nine genomic species with provisional designations are known. The genomic species groups are: Between 1 and 3; 6; Close to rpoB and gyrb; 13TU; 13BJ/14TU; 14BJ; 15BJ; 15TU; 16; and 17. In a recent study, Acinetobacter grimontii was reclassified as a later synonym of Acinetobacter junii (Vaneechoutte et al., 2008). Most members of the genus Acinetobacter have been isolated from clinical specimens. However, some species were isolated from environmental sources such as activated sludge, wetlands, forest soil, seawater, dump sites, wastewater, cotton and soil (Nishimura et al., 1988; Di Cello et al., 1997; Carr et al., 2003; Kim et al., 2008; Vaneechoutte et al., 2009; Anandham et al., 2010; Vaz-Moreira et al., 2011; Malhotra et al., 2012). The isolate HITLi 7T was isolated from the surface water of the Songhua River near Harbin in north-east China. A pure culture of HITLi 7T at 2 °C could remove ammonium at the rate of 0.04 mg N l−1 h−1 when the initial NH4+ N concentration was 5 mg l−1. The activity of ammonium monoxygenase (AMO) in strain HITLi 7T was about 8 nmol O2 min−1. The strain had nitrification ability. Based on the taxonomic data in this study, we propose that strain HITLi 7T represents a novel species with the suggested name, Acinetobacter harbinensis sp. nov.

Surface water samples were collected from the Songhua River near Harbin city, China (130° 10’ N 46° 40’ E), and incubated in an isolation medium at 2 °C for 14 days. The isolation medium consisted of 0.5 g NH4Cl l−1, 1.0 g CH3COONa l−1, 0.05 g MgSO4.7H2O l−1, 0.2 g KH2PO4 l−1, 0.12 g NaCl l−1, 0.01 g MnSO4.4H2O l−1, 0.01 g FeSO4 l−1 (pH 7.2). The culture was serially diluted with distilled water and ninefold dilutions were plated onto isolation agar medium. The plates were incubated at 2 °C for 3–5 days. HITLi 7T was the isolate that appeared on the isolation medium with heterotrophic nitrification and was subsequently stored at −80 °C as 10% (v/v) glycerol suspensions. After the optimum growth temperature for the strain was determined, cultures were routinely maintained on nutrient agar medium at 20 °C.
The 16S rRNA gene was amplified by PCR using a pair of universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTATCCAGC C-3') (Xing et al., 2006). PCR products were purified using an Agarose Gel DNA Purification kit TaKaRa Biotechnology). Sequencing was performed by TaKaRa Biotechnology (Dalian, China). 16S rRNA gene sequences of strain HITLi 7T showed the highest similarity (98.4%) to *Agarose Gel DNA Purification kit TaKaRa Biotechnology* (Xing et al., 2015). The maximum-likelihood tree showed the novel strain clustered with universal primers, 27F (5'–9) and 1541R (5'–9). The 16S rRNA gene was amplified by PCR using a pair of primers #1541R (5'–9) with other related species of the genus *Acinetobacter*. The 16S rRNA gene sequences of all known genomic species of the genus *Acinetobacter* were retrieved from GenBank and aligned with 16S rRNA gene sequences of strain HITLi 7T using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were reconstructed with neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods using the program MEGA6.0 (Tamura et al., 2013), with bootstrap values based on 1000 replications (Felsenstein, 1985). Phylogenetic analysis based on the neighbour-joining method revealed that HITLi 7T formed a clade from the cluster containing *A. beijerinckii NIPH 838* (Fig. 1). However, the maximum-parsimony tree and maximum-likelihood tree showed the novel strain clustered with *A. lwofii DSM 2403* and Genomic species 6 ATCC 17979 (Fig. S1 and Fig. S2, available in the online Supplementary Material).

The sequences of the *rpoB* gene were amplified by using two sets of primers: zone 1 spanning nucleotide positions 2916–3267 and zone 2 spanning nucleotide positions 3263–3773. Amplification was performed with the methods described previously (La Scola et al., 2006; Nemec et al., 2009). The *gyrB* gene sequences of strain HITLi 7 were amplified using the PCR primers PU-1E and APPr (Yamamoto & Harayama, 1996; Yamamoto et al., 1999). Direct sequencing of the PCR fragments was performed using primers M13 reverse or M13(−21). The PCR products of *rpoB* and *gyrB* genes were submitted to Sangong Biotech (Shanghai, China) for sequencing. The partially overlapping sequences encompassing the complete *rpoB* gene were combined into a single one with sequence assembler software (Applied Biosystems). The nucleotide sequences of *gyrB* and concatenated *rpoB* genes were analysed using MEGA6.0 software (Tamura et al., 2013). Sequence similarities were estimated based on the model of Jukes & Cantor (1969) and a dendrogram of estimated phylogenetic relationships was created using the neighbour-joining method.

The *rpoB* gene sequence of strain HITLi 7T was 861 bp (region 2915–3775), which did not show significant similarity (81.4–84.1%) with other type strains of recognized species and genomic species of the genus *Acinetobacter*. The neighbour-joining tree (Fig. 2) showed that the strain formed only a 53% bootstrap cluster with *Acinetobacter gerneri*. The *gyrB* gene sequence was 909 bp, which showed 76.9–81.4% similarity with other type strains of recognized species and genomic species of the genus. Other sequences used for *gyrB* sequence comparisons were retrieved from GenBank. The neighbour-joining tree is shown in Fig. 3. Strain HITLi 7T did not show high bootstrap clusters with species of the genus *Acinetobacter*.

The cell morphology of strain HITLi 7T was examined using light microscopy (CX31, Olympus) and transmission electron microscopy (H-9500, Hitachi). Gram-staining was performed as described by Gerhardt et al. (1994). Growth at various temperatures (2, 6, 8, 10, 15, 20, 25, 30, 35, 37, 41 and 44 °C) was tested in a beef extract-peptone medium solution in a water bath (Bouvet & Grimont, 1986). Oxidase activity was examined by oxidation of 1% (w/v) benzidine and tetramethyl-p-phenylenediamine (Deibel & Evans, 1960; Tarrand & Gröschel, 1982). Utilization of citrate and other carbon sources, aerobic acid production from glucose and gelatin hydrolysis were detected with methods described by Nemec et al. (2009). Phenotypic analysis was carried out using GNIII (Biolog), and API ZYM and API Coryne systems (bioMérieux) according to the manufacturers’ instructions. The temperature was 30 °C unless indicated otherwise.

Strain HITLi 7T was a Gram-stain-negative, catalase-positive, oxidase-negative, aerobic, non-motile, pleomorphic coccobacillus (0.7–1.3 μm × 0.8–1.4 μm). Colonies on nutrient agar were milky-white, circular, opaque and slightly convex; colonies were 0.2–0.6 mm in diameter after 16h and reached 1.2–2.0 mm in diameter after 48 h at 20 °C. Growth occurred at temperatures ranging from 2 to 35 °C. Good growth occurred at 8–20 °C in 1 day. No growth occurred at 37 °C or above in 7 days. Physiological and biochemical characteristics, metabolic properties and substrate utilization of strain HITLi 7T are given in detail in Tables S1, S2 and S3, and in the species description below.

The phenotypic properties of strain HITLi 7T and other related species of the genus *Acinetobacter* are shown in Table 1. Several phenotypic characteristics could be used to separate strain HITLi 7T from recognized species of the genus. Strain HITLi 7T could be differentiated from many species listed in Table 1 by its inability to grow at 37 °C. Strain HITLi 7T could be separated from *Acinetobacter gyllenbergii*, *A. haemolyticus* and *A. venetianus* by its inability to hydrolyse gelatin. The strain could be distinguished from *Acinetobacter baylyi*, *A. bereziniae*, *A. calcoaceticus*, *A. gerneri*, *A. guillouiae*, *A. gyllenbergii*, *A. radioreisens* and *A. soli* by its inability to utilize adipate and benzoate. The strain could be separated from *A. junii*, *A. lwofii*, *A. parvus*, *A. schindleri*, *A. ursingii*, *A. bouvetii* and *A. puyangensis* by its assimilation of β-alanine and malonate. The most useful characteristics for differentiating strain HITLi 7T from other species of the genus *Acinetobacter* were the inability to grow at 37 °C, hydrolyze gelatin, and assimilate adipate and benzoate, and utilization of β-alanine and malonate. Strain HITLi 7T showed unusual growth at 2 °C.

Fatty acid methyl esters were prepared according to the protocol of the Sherlock Microbial Identification System (MIDI) and analysed by GC (model 6890, Hewlett
Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. The position of strain HITLi 7T is shown with respect to other closely related species of the genus Acinetobacter. The tree was reconstructed using the neighbour-joining method. Moraxella lacunata ATCC 17967T was used as an outgroup. Numbers at nodes indicate bootstrap values, expressed as percentages of 1000 replications; only values >50% are shown. Bar, 0.01 changes per nucleotide position.
Packard) using Microbial Identification software (Sasser, 2001). Cells were grown on R2A for 48h at 30 °C (Anandham et al., 2010). The major fatty acids of HITLi 7T were 9-octadecenoic acid (C_{18:1}^\text{cis-9}, 41.92 %) and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}^\text{cis-7c}, 32.97 %). Strain HITLi 7T contained small amounts of C_{10:0} (2.98 %), C_{12:0} (3.37 %), C_{12:0} 3-OH (3.90 %), C_{16:0} (7.11 %) C_{18:0} (2.41 %) and C_{18:1}^\text{cis-7c} (4.82 %). The fatty acid profile was distinct from other related species of the genus *Acinetobacter* (Table 2).

Genomic DNA was extracted and purified using a Bacterial Genomic DNA Extraction kit (TaKaRa Biotechnology) according to the manufacturer’s instructions. The G+C content of the DNA was determined and DNA–DNA
hybridization was performed by the China General Microbiological Culture Collection Center (Institute of Microbiology, Chinese Academy of Science, China). The DNA G+C content was determined by using a Lambda35 UV/VIS Spectrophotometer (PerkinElmer) with *Escherichia coli K-12* as the reference. The DNA samples were diluted with 0.16 SSC (4.41 g l⁻¹ sodium citrate, 8.76 g l⁻¹ sodium chloride, pH 7.0) solution until the OD₂₆₀ value reached 0.3–0.4. The OD₂₆₀ value at 25°C was recorded. After that, the temperature was increased from 65°C to 95°C at 2°C min⁻¹. The temperature was controlled by a PTP-1 Peltier system. When the value of OD₂₆₀ appeared to increase, the temperature and OD₂₆₀ data were recorded and plotted. According to the denaturation curve, the Tₘ value was calculated. G+C (mol%) = G+C₇₄ (mol%) + 2.08 × (Tₘ − Tₘ₇₄). For identifying strain HITLi 7ᵀ from Genomic species 6 ATCC 17979, the DNA–DNA hybridization was analysed using a Lambda35 UV/VIS Spectrophotometer (PerkinElmer). According to the DNA G+C content, the optimal renaturation temperature (TOR) was calculated. TOR = 0.51 × G+C (mol%) + 47. The DNA samples were sheared to 2–5 × 10⁶ Da fragments with ultrasound (40W) for 24 min with stopping 3 s per 6 s. The DNA samples were diluted with 0.16 SSC solution until the OD₂₆₀ value reached 0.1–0.2. The OD₂₆₀ solution was used until the OD₂₆₀ value reached 1.8–2.0. The OD₂₆₀ of each sample was the same for hybridization. DNA (400 μl) of each bacterium was prepared separately in Eppendorf tubes. DNA (200 μl) of each bacterium was mixed in one EP tube for hybridization. The prepared DNA samples were denatured at 100°C for 15 min. After that, the temperature decreased to TOR. The total reaction was finished in 30 min. The results were recorded and analysed with UV Winlab software (PerkinElmer). The hybridization value was calculated according to the following equation:

\[
(H)\% = 4V_a - \frac{(V_{ab} + V_{b})}{2\sqrt{V_aV_b}} \times 100\%
\]

Where (H) is the hybridization value; V is the renaturation value; a is strain HITLi 7ᵀ; b is the strain Genomic species 6 ATCC 17979 and m is the mixed sample.

**Fig. 3.** Rooted neighbour-joining tree based on *gyrB* gene sequences showing the relationship between strain HITLi 7ᵀ and other members of the genus *Acinetobacter*. The sequence of *Escherichia coli* ATCC 25922 was used as an outgroup. Bootstrap values >50% (based on 1000 resamplings) are shown. Bar, 0.05 changes per nucleotide position.
Table 1. Differential phenotypic properties of strain HITLi 7T and related species

| Taxa: 1, strain HITLi 7T; 2, Acinetobacter baylyi; 3, A. beijerinckii; 4, Acinetobacter berceniariae; 5, Acinetobacter bouvetii; 6, Acinetobacter calcoaceticus; 7, A. gerneri; 8, Acinetobacter guillouiae; 9, Acinetobacter gillegemii; 10, Acinetobacter haemolyticus; 11, Acinetobacter johnsonii; 12, A. junii; 13, A. Iwaffii; 14, Acinetobacter parvus; 15, Acinetobacter puyangensis; 16, Acinetobacter radiotolerans; 17, Acinetobacter schindleri; 18, Acinetobacter soli; 19, Acinetobacter ursingii; 20, Acinetobacter venetianus. +, All strains positive; −, all strains negative; V +, 85–99 % of strains positive; V, 16–84 % of strains positive; V −, 1–15 % of strains positive; ND, not determined. All tests were conducted using similar methods. Data for reference taxa are given by Nemec et al. (2009, 2010, 2011) and Li et al. (2013). |

The results showed that the DNA G+C content of strain HITLi 7T was 45.5 mol%. The DNA–DNA hybridization value of HITLi 7T with Genomic species 6 ATCC 17979 was 25.5 %, indicating that strain HITLi 7T represented a species distinct to Genomic species 6.

Unfortunately, only one isolate was included in this study, resulting in lack of information about phenotypic variability within this species. However, the genotypic and phenotypic characteristics clearly distinguished strain HITLi 7T from other species within the genus Acinetobacter. Based on the results presented, a novel species, Acinetobacter harbinensis sp. nov. with the type strain HITLi 7T, is proposed.

Description of Acinetobacter harbinensis sp. nov.

Acinetobacter harbinensis (har.bin.en’sis. N.L. masc. adj. harbinensis pertaining to Harbin, north of China, from where the type strain was isolated).

Cells are Gram-stain-negative, strictly aerobic, catalase-positive, oxidase-negative, non-motile coccobacilli, capable of growth in mineral media with acetate as the sole carbon source and ammonia as the sole source of nitrogen. Colonies on nutrient agar are milky white, circular, opaque and slightly convex; colonies are 0.2–0.6 mm in diameter after 16 h and reach 1.2–2.0 mm in diameter after 48 h at 20 °C. Growth occurs at temperatures ranging from 2 to 35 °C. Good growth occurs at 8–20 °C in 1 day. No growth occurs at 37 °C or above in 7 days, with unusual growth at 2 °C. Cells grow at a pH range from pH 6 to 8.5 (optimum pH 7.2). Growth is inhibited by >4 % NaCl (w/v). Acid is not produced from D-glucose; gelatin is not hydrolysed. β-Alanine, azelate, ethanol, L-glutamate, DL-lactate and malonate can be utilized as the sole source of carbon with growth visible within 6 days (generally 2 days) of incubation. No growth on adipate, 4-aminobutyrate, L-arabinose, L-arginine, L-aspartate, benzoate, citrate (Simmons’), D-glucuronate, D-glucose, glutarate, L-histidine, 4-hydroxybenzoate, D-malate, putrescine, D-ribose or L-tyrtrate occurs within 10 days. The major fatty acids are 9-undecenoic

Table 2. Fatty acid profiles of strain HITLi 7T and other related species of the genus Acinetobacter

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>–</td>
<td>–</td>
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<td>7.5</td>
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<tr>
<td>C12:0 2-OH</td>
<td>4.5</td>
<td>3.4</td>
<td>6.2</td>
<td>1.2</td>
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<td>–</td>
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<tr>
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<td>3.90</td>
<td>3.0</td>
<td>5.8</td>
<td>9.3</td>
<td>7.9</td>
<td>4.7</td>
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<td>–</td>
<td>–</td>
<td>1.3</td>
</tr>
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<td>–</td>
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<td>–</td>
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<td>–</td>
<td>1.6</td>
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<tr>
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<td>42.4</td>
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<td>–</td>
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<tr>
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<td>30.7</td>
<td>14.1</td>
<td>33.7</td>
<td>40.2</td>
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</table>

*Summed features contain the following fatty acids: summed feature 3, C14:0, C16:1ω9c and/or C16:1iso I; summed feature 4, iso-C15:0 2-OH and/or C16:1ω7c.
acid (C_{18:1ω9c}) and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1ω7c}).

The type strain, with nitrifying ability, is HITLi 7^T (=CGMCC 1.12528^T=KCTC 32411^T), which was isolated from the Songhua River, Harbin, People’s Republic of China. The DNA G+C content of the type strain is 45.5 mol%.

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References


