**Acinetobacter larvae sp. nov., isolated from the larval gut of Omphisa fuscidentalis**

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**Abstract**

A Gram-stain-negative, non-spore-forming, non-motile and aerobic coccobacilli-shaped strain, designated BRTC-1<sup>T</sup>, was isolated from the gut of *Omphisa fuscidentalis* Hampson, which is a larva of a moth and was collected from Xishuangbanna Dai Autonomous Prefecture in China. The isolate was found to grow at NaCl concentrations of 0–5 % (w/v) (optimum: 0 %), 10–45 °C (optimum: 30–35 °C) and pH 5.0–9.0 (optimum: pH 6.0) on tryptic soy agar. Analysis of the 16S rRNA gene sequence indicated that the isolate belonged to the genus *Acinetobacter* and was most closely related to *Acinetobacter rudas LMG 26107<sup>T</sup>, *Acinetobacter guillouiae* LMG 988<sup>T</sup> and *Acinetobacter berezinae* LMG 1003<sup>T</sup> with 96.4, 96.3 and 96.3 % sequence similarity, respectively. The comparative sequence analyses of the rpoB and gyrB genes showed that BRTC-1<sup>T</sup> is distant from the species of the genus *Acinetobacter* with validly published names (<84.0 and <82.0 % similarity, respectively). The average nucleotide identity and digital DNA–DNA hybridization values (<77.0 and <22.8 %, respectively) between the whole-genome sequence of BRTC-1<sup>T</sup> and those of the known taxa were far below the thresholds used to discriminate bacterial species. The major fatty acids were determined to be C<sub>16:0</sub>, C<sub>18:1ω9c</sub> and C<sub>16:1ω7c/iso-C<sub>15:0</sub> 2-0H</sub>. The respiratory quinone was Q-9. The polar lipids were found to be diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, five phospholipids and one phosphatidylcholine. Based on its phenotypic and chemotaxonomic characteristics from this study, the isolate is concluded to represent a novel species of the genus *Acinetobacter*, for which the name *Acinetobacter larvae* sp. nov. is proposed. The type strain is BRTC-1<sup>T</sup> (=ACCC 19936<sup>T</sup>≡JCM 31367<sup>T</sup>).

The genus *Acinetobacter* was originally established by Bis sou and Prévot [1]. The type species is *Acinetobacter calcoaceticus*. At the time of writing, the genus consists of more than 40 species with validly published names (www.bacterio.net/acinetobacter.html). Previously described species of the genus *Acinetobacter* were isolated from various habitats, such as *Acinetobacter baylyi* from activated sludge [2], *Acinetobacter beijerinckii* from humans [3], *Acinetobacter brisouii* from a wetland [4], *Acinetobacter apis* from the intestinal tract of a honey bee [5], *Acinetobacter gandensis* from horses and cattle [6] and *Acinetobacter lac taeus* from iceberg lettuce [7]. Members of the genus *Acinetobacter* are non-motile, strictly aerobic, oxidase-negative, catalase-positive, Gram-stain-negative coccobacilli [8] and have genomic DNA G+C contents within the range of 38–47 mol% [9]. *Omphisa fuscidentalis* Hampson is a larva of a moth belonging to the family Pyralidae which is found in northern Thailand, Laos, Myanmar and China. The fifth instar larvae enter diapause and remain inside the internode of bamboo culm for 9 months from September until the following June [10]. While investigating the microbial community in the gut of *O. fuscidentalis* Hampson, a *Acinetobacter*-like strain, designated BRTC-1<sup>T</sup>, was isolated. By using a polyphasic taxonomic approach, we conclude that strain BRTC-1<sup>T</sup> represents a novel species of the genus *Acinetobacter*, for which the name *Acinetobacter larvae* sp. nov. is proposed.

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**Keywords:** 16S rRNA gene; rpoB gene; gyrB gene; whole-genome comparison; metabolic and physiological features.

**Abbreviations:** ACCC, Agricultural Culture Collection of China; ANIb, average nucleotide identity based on BLAST; dDDH, digital DNA–DNA hybridization with recommended parameters and/or default settings.

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The DDBJ/ENA/GenBank accession numbers for the 16S rRNA, gyrB and rpoB gene sequences of *Acinetobacter larvae* BRTC-1<sup>T</sup> are KU560468, KU981067 and KU837228, respectively. The whole-genome PacBio RS II project for *A. larvae* BRTC-1<sup>T</sup> has been deposited at DDBJ/ENA/GenBank under the accession number CP016895.

Four supplementary figures and one supplementary table are available with the online Supplementary Material.
Strain BRTC-1T was isolated from the gut of O. fuscidentalis Hampson collected from Xishuangbanna Dai Autonomous Prefecture in China. After dissection of O. fuscidentalis Hampson, the gut was separated, mashed and suspended in sterilized water, then serially diluted and spread on tryptic soy agar (TSA) medium (BD/Difco) plates, and aerobic incubation was performed at 30 °C for 36 h. Strain BRTC-1T was isolated, and its single colony was picked and purified by repeatedly streaking on fresh TSA solid plates and then stored at −80 °C as 10 % (v/v) glycerol suspensions. Biomass for chemotaxonomic and molecular studies was obtained by cultivation in shaking flasks with tryptic soy broth (TSB) medium (BD/Difco) at 30 °C for 2 days. The proposed minimal standards for the description of aerobic, non-motile cocccobacilli [11] were followed.

The complete 16S rRNA gene sequence (1524 bp) was obtained from the genome of BRTC-1T by using the EzTaxon-e service [13] and CLUSTALW software [14], respectively. To confirm the relatedness of strain BRTC-1T to the genus Acinetobacter, and its separation from all species of this genus, comparative sequence analysis of the rpoB and gyrB genes was also performed. The sequence of the rpoB gene was amplified by using two sets of primers: zone 1 spanning nucleotide positions 2916–3267 and zone 2 spanning nucleotide positions 3263–3773 [15]. Amplification was performed with the methods described previously [15]. The gyrB gene sequences of strain BRTC-1T were amplified using the PCR primers PU-1E and APuR [16]. Direct sequencing of the PCR fragments was performed using primers M13 reverse and M13 (-21). The PCR products of rpoB and gyrB genes were sequenced by the Tsingke Company (Chengdu, China). The nucleotide sequences of the rpoB and gyrB genes were compared with reference sequences available in the GenBank/EMBL/DDBJ database using BLAST from NCBI (www.ncbi.nlm.nih.gov). Phylogenetic trees were reconstructed via the neighbour-joining method as described by Saitou et al. [17], the maximum-parsimony method [18] and the maximum-likelihood method [19] with the MEGA6.0 program package [20] and evaluated by bootstrap analysis with 1000 replications as described by Felsenstein [21].

The 16S rRNA gene sequences of strain BRTC-1T showed the highest similarity (96.4 %) to Acinetobacter ruidis LMG 26107T. The topology of phylogenetic trees, built by the neighbour-joining, the maximum parsimony and the maximum-likelihood methods, support the conclusion that strain BRTC-1T forms a stable clade with the type strain of A. ruidis LMG 26107T (Fig. 1). The rpoB gene (860 bp) and gyrB gene (909 bp) sequences of strain BRTC-1T showed less than 84 and 82 % similarity, respectively, with other type strains of recognized species and genomic species of the genus Acinetobacter (Figs S1 and S2, available in the online Supplementary Material). Based on genetic data analyses, strain BRTC-1T should be considered a representative of a novel species of the genus Acinetobacter.

Genomic DNA was isolated from strain BRTC-1T by the modified CTAB (cetyltrimethylammonium bromide) method [22]. Whole-genome sequencing of BRTC-1T was performed by Novogene (Beijing, China) using the PacBio RS II platform with the Single Molecule Real Time (SMRT) [23]. Qualified genomic DNA was prepared using an E.Z.N. A.TM Bacterial DNA Kit (Omega Bio-Tek). A SMART bell sequencing library with a 10 kb insert size was prepared using the SMARTbell Template Prep Kit (PacBio RS II). The data from a SMRT cell was filtered to exclude low quality reads by using SMRT Analysis (version 2.3.0) and then assembled by software SMRT portal [24] to obtain one contig without gaps (size: 3741 098 bp, number of proteins: 3149). The DNA G+C content was 41.6 %, which is within the range reported for members of the genus Acinetobacter (38–47 mol%) [9]. The complete genome sequence of BRTC-1T was compared with those representing nearly all known species of the genus Acinetobacter using the average nucleotide identity based on BLAST (ANIb) and digital DNA–DNA hybridization (dDDH) parameters. JSpecies [25] (www imedea. uib.es/jspecies) and the GGDC web server [26] (http://ggdc.dsmz.de) programs were respectively used to calculate ANIb and dDDH values with the recommended parameters and/or default settings. The results are summarized in Table S1. The ANIb and dDDH values were equal to or lower than 77.09 % (Acinetobacter guillouiae CIP 63.46T) and 22.8 % (Acinetobacter junii CIP 64.5T), respectively, which are far below the thresholds recommended to discriminate bacterial species [7]. The whole-genome comparison supported that strain BRTC-1T represents a novel species of the genus Acinetobacter.

Cellular morphological features of an exponentially growing culture of strain BRTC-1T were observed by optical microscopy (80i; Nikon) and a transmission electron microscope (JSM-7500F; JEOL). The Gram-staining reaction, catalase, oxidase and urease activities, nitrate reduction, Voges-Proskauer test, aesculin hydrolysis, and indole and H2S production were determined as described by Smibert and Krieg [27]. Aerobic acid production from glucose, utilization of citrate (Simmons’) and gelatin hydrolysis were detected with methods as described by Nemec et al. [3]. Phenotypic profiling was performed, using the API 20E, API 20NE and API ZYM panels (bioMérieux), following the instructions of the manufacturer, and the results were observed after 48 h. Unless stated, all tests were performed under the optimal conditions for each strain. To confirm the results obtained, the assimilation of another 32 carbon sources was performed using the basal mineral medium of Cruze et al. [28] supplemented with 0.1 % (w/v) of each carbon source as described by Nemec et al. [3]. Cell motility was tested in TSB medium with 0.4 % agar. Growth under anaerobic conditions was tested in TSB medium in Hungate tubes filled with oxygen-free N2 at 30 °C for 3 days. The formation of endospores was tested on TSB agar supplemented with 5
mg MnSO$_4$ 1$^{-1}$ as described by Logan et al. [29]. Growth at temperatures of 4, 10, 15, 20, 30, 37, 40, 45, 48, 50 and 55 °C was investigated in TSB medium as described by Bouvet and Grimont [30]. Growth at pH 4, 5, 6, 7, 8, 9, 10 and 11 was examined in TSB medium at 30 °C, and the desired pH values for each test were adjusted according to Wang et al. [31]. NaCl concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 % (w/v) were produced in medium prepared according to the formula of TSB but varying the addition of NaCl. The growth was monitored by measuring the optical density at 600 nm using a spectrophotometer (UV1800; Shimadzu). Haemolytic activity was tested on Columbia agar with 5 % NaCl (optimum 0 %), at temperatures varying from 10 to 45 °C. The utilization of 4-aminobutyrate, L-aspartate, trans-aconitate and putrescine differed the isolate from A. rudis. The strain could be differentiated from A. guillouiae and Acinetobacter bereziniae in that it could not utilize azelate or histamine. Azelate was utilized by all the reference strains but not utilized by the strain BRTC-1T. In addition, only trans-aconitate, L-ornithine, putrescine were utilized by the new isolate.

The polar lipids were extracted and separated on a silica gel plate (10 × 10 cm; Merck) and further analysed as described by Minnikin et al. [32]. Molybdophosphoric acid was used to reveal the total polar lipids, ninhydrin for aminolipids, normal phase thin-layer chromatography (TLC) for phospholipids. The polar lipids were determined to be diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), five phospholipids (PL) and one phosphatidylcholine (PC) (Fig. S4). The presence of PE, PG, DPG and PC was similar to the recently described strains of the genus Acinetobacter [33, 34]. The isolate shared most of the spots (PL1–3, PL5, PE, DPG, PG, PC) with the related strain A. rudis DSM 30006T. However, aminophospholipid and aminolipid possessed by A. rudis

![Phylogenetic tree](image)

**Fig. 1.** Neighbour-joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain BRTC-1T and its close relatives. Genbank accession numbers are given in parentheses. Bootstrap percentages, generated from 1000 resamplings, ≥50 % are indicated at branch points. Filled circles indicate branches also recovered by the maximum-parsimony and maximum-likelihood methods. Bar, 5 substitutions per 1000 nucleotide positions.
were absent in strain BRTC-1\textsuperscript{T}. Quinones were extracted according to the method described by Dadhwal et al. \cite{35} and analysed by reverse-phase TLC, according to the methods of Collins and Jones \cite{36}. The predominant respiratory quinone was identified as Q-9, which was in line with all members of the genus Acinetobacter \cite{33, 37}. For determination of cellular fatty acid composition, strain BRTC-1\textsuperscript{T}, A. rudis LMG 26107\textsuperscript{T}, A. guillouiae LMG 988\textsuperscript{T} and A. bereziniae LMG 1003\textsuperscript{T} were incubated on TSA at 30 °C for 24 h. The fatty acid methyl esters were obtained from the cells collected from the plates. The identification and quantitation were performed using the Sherlock Microbial Identification System with the standard MIS Library Generation Software (version 6.0 and date 4; Microbial ID). The analyses of cellular fatty acids were conducted as described by Sakamoto et al. \cite{38}. The major fatty acids of strain BRTC-1\textsuperscript{T} were C\textsubscript{16:0} (16.9 %), C\textsubscript{18:1}\textomega7c (40.6 %) and C\textsubscript{16:1}\textomega7c\textslash\textomega10:0 (15.3 %). Although the majority of the cellular fatty acid composition of strain BRTC-1\textsuperscript{T} was generally similar to those of the reference species, there were differences in the proportions of the components presented. The predominant fatty acid of BRTC-1\textsuperscript{T} were C\textsubscript{18:1}\textomega7c (40.6 %), which was present in greater amounts than in A. rudis LMG 26107\textsuperscript{T} (36.8 %), A. bereziniae LMG 1003\textsuperscript{T} (35.7 %) and A. guillouiae LMG 988\textsuperscript{T} (38.0 %). The proportion of summed feature 3 (C\textsubscript{16:1}\textomega7c\textslash\textomega10:0 2-OH) (15.3 %) of BRTC-1\textsuperscript{T} was lower than found in A. rudis LMG 26107\textsuperscript{T} (25.7 %), A. bereziniae LMG 1003\textsuperscript{T} (26.4 %) and A. guillouiae LMG 988\textsuperscript{T} (27.5 %). A significant difference was observed in terms of the percentage of C\textsubscript{17:0} 10-methyl, C\textsubscript{18:2}\textomega6c and C\textsubscript{18:1} between BRTC-1\textsuperscript{T} and related species of this genus (Table 2). All these chemotaxonomic data suggest that strain BRTC-1\textsuperscript{T} represents a novel species of the genus Acinetobacter.

**DESCRIPTION OF ACINETOBACTER LARVAE SP. NOV.**

Acinetobacter larvae (lar’vae. L. gen. n. larvae of a larva).

Colonies are milky-yellow, circular, smooth and convex with a diameter of 1.0–3.0 mm after 3 days of incubation at 30 °C on TSB medium. Cells are Gram-stain-negative, strictly aerobic, non-motile coccobacilli with a size of 0.2–0.8 µm in width and 0.5–1.1 µm in length. Catalase-positive and oxidase-negative. Grows with 0–5 % (w/v) NaCl (optimum 0 %), at temperatures varying from 10 to 45 °C (optimum 30–35 °C) and in the pH range 5.0–9.0 (optimum pH 6.0). Growth does not occur under anaerobic conditions, and nitrate is not reduced. Acid is not produced from D-glucose, gelatin is not hydrolysed. Haemolysis is not observed. Utilization of Simmons’ citrate (growth) is positive. Aesculin, casein and starch are not hydrolysed. H\textsubscript{2}S and indole are not produced. Glucose is not fermented. Voges-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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</thead>
<tbody>
<tr>
<td>Growth temperature (°C)</td>
<td>10–45</td>
<td>10–37</td>
<td>25–38</td>
<td>25–35</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>Azelate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>t-Aspartate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>t-Arginine</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>4-Aminobutyrate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>trans-Aconitate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>Malonate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>t-Ornithine</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
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<tr>
<td>t-Phenylalanine</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+*</td>
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<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
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<td>Trigonelline</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
<tr>
<td>Tricarballylate</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>–</td>
</tr>
</tbody>
</table>

*According to the literature, variable results for this test may be observed for members of this species.

Table 2. Cellular fatty acid composition of strain BRTC-1\textsuperscript{T} and related type strains of species of the genus Acinetobacter

Strains: 1. BRTC-1\textsuperscript{T}; 2. A. rudis LMG 26107\textsuperscript{T}; 3. A. bereziniae LMG 1003\textsuperscript{T}; 4. A. guillouiae LMG 988\textsuperscript{T}. All strains were cultivated on TSA at 30 °C for 24 h. Values are percentages of total fatty acids; components that made up <1 % of the total in all strains are not shown. TR, Trace; –, not detected. Predominant components are highlighted in bold type.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
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<th>3</th>
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<tbody>
<tr>
<td>C\textsubscript{10:0} 2-OH</td>
<td>TR</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{12:0} 6.6</td>
<td>5.3</td>
<td>8.2</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{12:0} 2-OH</td>
<td>2.4</td>
<td>2.5</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>C\textsubscript{12:0} 3-OH</td>
<td>6.2</td>
<td>5.3</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>iso-C\textsubscript{15:0}</td>
<td>TR</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{16:0} 16.9</td>
<td>17.8</td>
<td>15.3</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{16:0} 19.6</td>
<td>TR</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{17:0} 10-methyl</td>
<td>2.3</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C\textsubscript{17:0}</td>
<td>TR</td>
<td>TR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{18:0} 40.6</td>
<td>36.8</td>
<td>35.7</td>
<td>38.0</td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{18:0} 1.4</td>
<td>TR</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{18:0} 4.0</td>
<td>1.4</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
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<tr>
<td>Summed feature 3*</td>
<td>15.3</td>
<td>25.7</td>
<td>26.4</td>
<td>27.5</td>
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<tr>
<td>Summed feature 8*</td>
<td>3.0</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that cannot be separated by the MIDI System. Summed feature 3 contains C\textsubscript{16:1}\textomega7c and/or iso-C\textsubscript{15:0} 2-OH; summed feature 8 contains one or more of C\textsubscript{17:0}\textomega7c and C\textsubscript{18:1}\textomega6c.
Proskauer test is positive. Produces the enzymes esterase (C4), esterase lipase (C8), lipase (C14), and alkaline and acid phosphatases. Soluble carbon sources assimilated include acetate, ethanol, citrate, 4-aminobutyrate, L-aspartate, L-histidine, d-malate, malonate, d-lactate, trans-acetoin, glutarate, β-alanine, 4-hydroxybenzoate, L-ornithine, L-arginine, 2,3-butanedioin, benzoate, adipate and putrescine. No growth occurs on azelate, histamine, 2,3-butanediol, benzoate, adipate and putrescine. No growth occurs on trigonelline, tricarballylate or proline. The respiratory quinone is Q-9. The major polar lipids are phosphatidylglycerol and phosphatidylethanolamine.

The type strain is BRTC-1T (=ACCC 19936T =JCM 31367T), which was isolated from the larval gut of Omphisa fuscidentalis collected from Xishuangbanna Dai Autonomous Prefecture in China. The genomic DNA G+C content of the type strain is 41.6 mol%.

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

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