**Rhizobium borbori** sp. nov., aniline-degrading bacteria isolated from activated sludge

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Three aniline-degrading bacteria, strains DN316T, DN316-1 and DN365, were isolated from activated sludge. According to 16S rRNA gene sequence-based phylogenetic analysis, the isolates belonged to the genus *Rhizobium*, with *Rhizobium* (=Agrobacterium) *radiobacter* LMG 140T as the closest relative, with 96.5% sequence similarity. Phylogenetic analysis of the representative strain DN316T using sequences of the *glnA*, *thrC* and *recA* genes and the 16S–23S intergenic spacer region confirmed the phylogenetic arrangement obtained from analysis of the 16S rRNA gene. DNA–DNA relatedness between DN316T and *R. radiobacter* LMG 140T was 43.7%, clearly indicating that the representative strain DN316T represents a novel species. Phenotypic and biochemical characterization of the isolates and insertion sequence-PCR fingerprinting patterns showed several distinctive features that differentiated them from closely related species. The major components of the cellular fatty acids were C18:1 (57.10%), C16:0 (11.31%) and C19:0 cyclo 9c (10.13%). Based on our taxonomic analysis, the three isolates from activated sludge represent a novel species of the genus *Rhizobium*, for which the name *Rhizobium borbori* sp. nov. is proposed. The type strain is DN316T (=CICC 10378T =LMG 23925T).

The genus *Rhizobium* was described by Frank (1889). After a series of taxonomic changes that have been proposed since the 1980s, the genus *Rhizobium* consists of 43 species at the time of writing, including the recently described species *Rhizobium selenitireducens* (Hunter et al., 2007), *R. pisi* (Ramirez-Bahena et al., 2008), *R. oryzae* (Peng et al., 2008), *R. multihabitatium* (Han et al., 2008), *R. miiouense* (Gu et al., 2008), *R. alamii* (Berge et al., 2009), *R. alkalosoli* (Lu et al., 2009), *R. mesosinicum* (Lin et al., 2009) and *R. tibeticum* (Hou et al., 2009). Most of them have been isolated from nodules of leguminous plants or the roots of cereals as symbiotic or associated nitrogen-fixation partners, with the exception of the selenite-reducing *R. selenitireducens*, exopolysaccharide-producing *R. alamii*, polysaccharide-hydrolysing *Rhizobium cellosolyticum* and cyanide-degrading *Rhizobium dacejonense* (Berge et al., 2009; Garcia-Fraile et al., 2007; Hunter et al., 2007; Quan et al., 2005).

During the course of collection of micro-organisms from environmental sources, three pure bacterial cultures were obtained from aniline-polluted activated sludges. Their taxonomic position was determined by a polyphasic taxonomic analysis, and the results suggest that these strains belong to a novel *Rhizobium* species.

For many years, anilines have been among the most important industrially produced amines. They are used widely in the production of polyurethanes, rubber, azo dyes, drugs, photographic chemicals, varnishes and pesticides (Gheewala & Annachhatre, 1997; Kearney & Kaufman, 1969). Aniline is a harmful substance that pollutes the environment and seriously endangers human health; it has been included on a Chinese blacklist for priority control in environmental protection. Aniline is biodegraded mainly by micro-organisms in soils and water bodies, and many aniline-degrading strains have been reported, such as strains of *Delftia* (Boon et al., 2001; Liu et al., 2002; Zhang et al., 2008; Liang et al., 2005); *Pseudomonas* (Meyers, 1992; Fukumori & Saint, 1997;...
Bathe, 2004) and Acinetobacter (Takeo et al., 1998; Fujii et al., 1997). To the best of our knowledge, no rhizobia able to degrade aniline have been reported.

Strains DN316\textsuperscript{T}, DN316-1 and DN365, positive for aniline degradation, were isolated on MSA medium from activated sludge of a textile-printing wastewater-treatment plant, a municipal wastewater-treatment plant and a laboratory sequencing batch reactor, respectively. MSA medium contained:\begin{itemize}
\item 1.73 g L\textsubscript{2}HPO\textsubscript{4}, 1.73 g KH\textsubscript{2}PO\textsubscript{4}, 1.0 g NH\textsubscript{4}NO\textsubscript{3}, 0.1 g MgSO\textsubscript{4}, 0.03 g FeSO\textsubscript{4}. 7H\textsubscript{2}O, 0.03 g FeCl\textsubscript{3}, 0.02 g CaCl\textsubscript{2}. 2H\textsubscript{2}O, 0.03 g MnSO\textsubscript{4}. H\textsubscript{2}O, 0.5 g aniline and 16 g agar, pH 7.0. Activated sludge samples were shaken for 2 h on a rotary shaker at 250 r.p.m. to disperse them and then suspensions were serially diluted with 0.85% (w/v) NaCl. Each dilution (0.1 ml) was plated onto MSA medium.
\end{itemize}

After 10 days of incubation at 28 °C, isolated colonies were purified further on MSA agar until single colonies were observed under the microscope. Pure cultures were stored in 15% (w/v) glycerol at 4 °C for long-term preservation. The cryoprotecting medium used was 8% sucrose, 10% skimmed milk and 1.5% gelatin.

The concentration of aniline was determined by spectrophotometry. Overnight cultures in 5 ml Luria–Bertani (LB) medium (1.0 g tryptone, 5 g yeast extract, 10 g NaCl; pH 7.0) were used as inocula for degradation experiments. Pelleted cells, collected from 1 ml culture by centrifugation for 3 min at 8000 r.p.m., were washed and resuspended in 1 ml saline water (0.85% NaCl), 50 μl inoculum was mixed in 5 ml liquid MSA medium (MSA without agar; liquid MSA medium without inoculum was used as a control) and cultures were incubated at 28 °C with shaking at 175 r.p.m. (all assays were conducted in triplicate). After 60 h, cells were removed by centrifugation for 5 min at 8000 r.p.m. The A\textsubscript{450} of the coloured complex of aniline with N-(1-naphthyl)-ethylenediamine dihydrochloride was determined using a UV spectrophotometer (Beckman DU-640). Aniline-degrading activity (%) was calculated as \([A − B]/A \times 100\%\), where \(A\) is the initial concentration of aniline and \(B\) is the residual aniline concentration. The aniline-degradation activities of isolates DN316\textsuperscript{T}, DN316-1 and DN365 were respectively 28 ± 1.48, 32 ± 2.11 and 35 ± 1.87%.

Phenotypic tests including utilization of carbon sources, tolerance of salt and conditions for growth were performed according to the following methods. The isolates and reference strains were incubated aerobically on YMA (Vincent, 1970) and/or LB plates overnight at 28 °C. Gram staining was observed by normal light microscopy according to the Hucker method (Murray et al., 1994). Carbon-source utilization was tested with GN2 MicroPlates (Biolog). Overnight cultures were used to inoculate the GN2 MicroPlates according to the manufacturer’s instructions. Tolerance of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7% (w/v) NaCl was measured spectrophotometrically by following the OD\textsubscript{550} in YMA medium after 18–24 h of incubation at 28 °C. The pH range for growth was studied between pH 4.0 and 10.0 under the same test conditions. Resistance to dyes was investigated after 24 h of incubation at 28 °C on YMA medium containing the following dyes at the concentrations indicated (w/v): 0.03, 0.06 and 0.12% Amaranth, 0.019, 0.038 and 0.076% crystal violet and 0.035, 0.70 and 0.14% reactive brilliant blue X-BR. Results were scored as positive if the change in OD\textsubscript{550} was greater than 0.300 (Heyrman et al., 2002).

Cells of strain DN316\textsuperscript{T} were Gram-negative rods. The strain was negative for assimilation of starch and cellulose. The isolates had phenotypic properties consistent with their classification in the genus Rhizobium. Some phenotypic characteristics of the novel strains in comparison with strains of several Rhizobium species are shown in Table 1.

### Table 1. Major phenotypic features of strains DN316\textsuperscript{T}, DN316-1 and DN365 and strains of related Rhizobium species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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</thead>
<tbody>
<tr>
<td>Growth on LB medium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tolerance of 2% NaCl</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Growth at 37 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth at pH 10.0</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Utilization of sole carbon sources</td>
<td></td>
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<tr>
<td>D-Arabinose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Citrate</td>
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<td>−</td>
<td>−</td>
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<tr>
<td>Glycerol</td>
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<td>Methyl β-D-glucoside</td>
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<td>−</td>
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<td>Raffinose</td>
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<td>Xylitol</td>
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<td>L-Histidine</td>
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<td>L-Proline</td>
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<td>−</td>
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<td>Acetic acid</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>Tolerance of dyes</td>
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<td></td>
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<tr>
<td>Amaranth (0.06%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Crystal violet (0.038%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Reactive brilliant blue X-BR (0.07%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aniline-degrading ability</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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Table 2. Major fatty acids of strain DN316T and type strains of related Rhizobium species.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>C18:1ω7c</td>
<td>57.10</td>
<td>57.22*</td>
<td>53.41 (52.4*)</td>
<td>48.96 (73.2*)</td>
<td>51.45 (71.0*)</td>
</tr>
<tr>
<td>C16:0</td>
<td>11.31</td>
<td>4.08</td>
<td>9.05 (9.0)</td>
<td>11.28 (9.6)</td>
<td>9.77 (8.1)</td>
</tr>
<tr>
<td>C19:0 cyclo ω8c</td>
<td>10.13</td>
<td>13.91</td>
<td>17.87 (18.8)</td>
<td>16.72 (2.4)</td>
<td>17.65 (2.5)</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>7.81</td>
<td>1.32</td>
<td>4.73 (4.8)</td>
<td>5.73 (3.3)</td>
<td>5.06 (4.1)</td>
</tr>
<tr>
<td>C16:1ω7c iso-C15:0 2-OH</td>
<td>5.55</td>
<td>0.52</td>
<td>1.60 (1.6)</td>
<td>2.95 (4.0)</td>
<td>2.01 (6.2)</td>
</tr>
<tr>
<td>11-Methyl C18:1ω7c</td>
<td>2.36</td>
<td>4.70</td>
<td>0.23 (NR)</td>
<td>0.99 (NR)</td>
<td>0.41 (NR)</td>
</tr>
<tr>
<td>C18:1ω7c</td>
<td>1.24</td>
<td>7.83</td>
<td>0.17 (0.2†)</td>
<td>0.29 (1.5)</td>
<td>0.30 (NR)</td>
</tr>
<tr>
<td>C17:0 cyclo</td>
<td>1.14</td>
<td>0.15</td>
<td>1.56 (1.6)</td>
<td>2.54 (NR)</td>
<td>2.29 (NR)</td>
</tr>
<tr>
<td>C14:0 3-OH iso-C16:1 I</td>
<td>0.93</td>
<td>6.46‡</td>
<td>10.16 (8.2‡)</td>
<td>10.13 (6.0‡)</td>
<td>10.76 (6.9‡)</td>
</tr>
</tbody>
</table>

*Reported as summed feature 7 (C18:1ω7ciso-C15:0ω12t).
†Data from Jarvis et al. (1996); not reported by Quan et al. (2005).
‡Reported as summed feature 3 [defined as one or more of C12:0 (aldehyde), unknown ECL 10.928, iso-C16:1 I and C14:0 3-OH].

Previous studies have demonstrated that cellular fatty acid analysis is a useful tool for identifying unknown strains of rhizobia. Strain DN316T, R. radiobacter LMG 140T, R. rubi LMG 17935T and R. larrymoorei AF3.10T were incubated for 36 h in YMA medium at 28°C. Sample preparation was performed according to the method described by Song et al. (2000). Extraction and analysis of cellular fatty acid methyl esters were carried out as described by Xu et al. (2005). The fatty acid profile of representative strain DN316T contained predominantly C18:1ω7c (57.10%), C16:0 (11.31%) and C19:0 cyclo ω8c (10.13%) (Table 2). Minor fatty acids not included in Table 2 were C13:1 at 12–13% (0.49%), C12:0 3-OH (0.09%), C15:0 (0.11%), C17:0 ω8c (0.15%), C17:0 (0.18%), iso-C18:0 (0.14%) and C18:2ω6c 9c and/or anteiso-C18:1ω6c (0.16%). Fatty acid analysis showed that DN316T contained high levels of C18:1ω7c, which was consistent with the reference strains. The main differences were the smaller amount of C14:0 3-OH and/or iso-C16:1 I and the larger amount of C16:0 3-OH in comparison with the reference strains.

The 16S rRNA gene was amplified from genomic DNA as described by Zhang et al. (2007). PCRs were performed according to a previously described method (Hurek et al., 1997). Purified PCR products were sequenced (three replications) by Shanghai Invitrogen Biotechnology Co., Ltd, using the forward primer 27f (5′-AGAGTTTGATCCTGATCTCAAG-3′) and reverse primer 1522r (5′-AAGGAGGTATCCAGGAGGCA-3′). The nearly full-length 16S rRNA gene sequence was compiled using SeqMan software (DNASTAR). The sequences determined, together with related sequences of Rhizobium species and other related species of the Alphaproteobacteria selected from the GenBank database with the BLASTN program (Altschul et al., 1997), were aligned by using the CLUSTALW software (Thompson et al., 1994). Alignment gaps and ambiguous bases were excluded from similarity calculations. The tree topology was inferred by using the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was visualized and bootstrapped by using the TREECON software package with 100 replicates for evaluation (Van de Peer & De Wachter, 1994). Amplification and sequencing of the glnA, thrC and recA genes and the 16S–23S intergenic spacer (IGS) were performed according to previously described methods (Martens et al., 2007; Peng et al., 2002). Phylogenetic analysis of these sequences was conducted by the same procedure as used for the 16S rRNA gene sequence.

The 16S rRNA gene sequences of the three isolates were nearly identical, sharing more than 99.5% sequence similarity. Analysis of the 1353 bp 16S rRNA gene sequences demonstrated that the three isolates formed a separate clade with recognized species of the genus Rhizobium (Fig. 1). Their closest phylogenetic relatives were R. radiobacter LMG 140T, R. rubi LMG 17935T and R. larrymoorei AF3.10T, with 96.5, 96.1 and 96.0% 16S rRNA gene sequence similarity, respectively. Representative strain DN316T showed 95.9% 16S rRNA gene sequence similarity to the type strain of R. leguminosarum. These data clearly demonstrated that the strains were members of a genomic species that differed from defined Rhizobium species.
In recent years, several studies have shown that the rRNA genes of rhizobia may occasionally undergo lateral transfer and genetic recombination, resulting in sequence mosaicism. To overcome these limitations, sequence analysis of suitable housekeeping genes might be required for reliable classification (Eardly et al., 1996, 2005; Sullivan et al., 1996; van Berkum et al., 2003; Vinuesa et al., 2005). To confirm our taxonomic conclusions, phylogenetic analyses of the glnA, thrC and recA gene sequences of representative strain DN316T were performed, and indicated strongly that strain DN316T formed a compact phylogenetic cluster with type strains in the genus *Rhizobium* and was allocated to the genus *Rhizobium* (Supplementary Figs S1, S2 and S3, available in IJSEM Online). Representative strain DN316T has the closest phylogenetic relationships with the type strains of *R. radiobacter* and *R. rubi* (bootstrap values above 60%).

The glnA, thrC and recA gene sequences of representative strains DN316T were performed, and indicated strongly that strain DN316T formed a compact phylogenetic cluster with type strains in the genus *Rhizobium* and was allocated to the genus *Rhizobium* (Supplementary Figs S1, S2 and S3, available in IJSEM Online). Representative strain DN316T has the closest phylogenetic relationships with the type strains of *R. radiobacter* and *R. rubi* (bootstrap values above 60%).

16S–23S IGS sequence analysis, which is considered to be a useful tool for investigating the relatedness of closely related rhizobial strains (Han et al., 2008), confirmed the phylogenetic arrangements obtained from analyses of the glnA, thrC and recA and 16S rRNA genes (Supplementary Fig. S4). The 16S–23S IGS sequence of strain DN316T showed 88 % similarity to that of *R. radiobacter* and 86 % and to that of *R. rubi* DSM 6772T, strongly supported by a high bootstrap value (88 %).

Insertion sequence-PCR (IS-PCR) analysis was performed to evaluate the genotypic differences between strains DN316T, DN316-1 and DN365, *Rhizobium* species and other related species of the Alphaproteobacteria. Bootstrap values above 50 (from 100 replications) are indicated at branch points. Bar, 2 substitutions per 100 nucleotide positions.

**Fig. 1.** Phylogenetic positions based on 16S rRNA gene sequences of strains DN316T, DN316-1 and DN365, *Rhizobium* species and other related species of the Alphaproteobacteria. Bootstrap values above 50 (from 100 replications) are indicated at branch points. Bar, 2 substitutions per 100 nucleotide positions.
The six strains examined had between six and nine fragments of 250–5000 bp (Fig. 2). Strains DN316<sup>T</sup>, DN316-1 and DN365 had nearly identical bands, which differed distinctly from those of closely related species of the genus *Rhizobium*. This difference in genomic organization supports the conclusion that strain DN316<sup>T</sup> represents a separate species of the genus *Rhizobium*.

To obtain high-quality DNA, total genomic DNA from the isolates and reference strains was isolated and purified as described by Marmur (1961). DNA base composition of each strain was determined spectrophotometrically (De Ley et al., 1970). DNA from *Escherichia coli* K-12 was used as a standard for estimation of G+C content. The genomic DNA G+C content of strain DN316<sup>T</sup> was 60.9±9.7 mol% (mean±sd; n=3); those of strains DN316-1 and DN365 and *R. radiobacter* LMG 140<sup>T</sup> and *R. rubi* LMG 17935<sup>T</sup> were 61.1±3.8, 62.7±1.1, 58.6±3.2 and 62.3±4.5 mol%, respectively, which were in the range expected for members of the genus *Rhizobium* (57–66 mol%; Young et al., 2001).

The threshold of 70% DNA–DNA relatedness for bacterial species delineation has been used in the description of many rhizobial species, such as *Mesorhizobium ciceri* (Nour et al., 1994) and *Rhizobium hainanense* (Chen et al., 1997). DNA–DNA relatedness was determined by the initial renaturation rate method (De Ley et al., 1970) in 2×SSC as modified by Tan et al. (2001). Hybridization experiments were carried out under optimal conditions, calculated from the melting temperature (T<sub>m</sub>) based on the DNA G+C content of each test strain, and stringent conditions (75 °C) (all tests were performed in triplicate). DNA–DNA relatedness of 96 and 99% was obtained between strain DN316<sup>T</sup> and isolates DN316-1 and DN365, indicating that they represent the same genomic species. DNA–DNA hybridization experiments revealed that strain DN316<sup>T</sup> showed only 43.7±2.8, 39.6±3.2 and 32.3±1.02% reassociation with *R. radiobacter* LMG 140<sup>T</sup>, *R. rubi* LMG 17935<sup>T</sup> and *R. larrymoorei* AF3.10<sup>T</sup>, respectively. These results indicated that the genomic species represented by strain DN316<sup>T</sup> was different from closely related species. The DNA G+C content and DNA–DNA hybridization results support the conclusion that strain DN316<sup>T</sup> represents a novel species within the genus *Rhizobium*.

All the results obtained based on a polyphasic taxonomic study demonstrate that the three novel isolates can be differentiated genotypically and phenotypically from their nearest phylogenetic neighbours. We believe that the aniline-degrading isolates represent a novel species of the genus *Rhizobium*, *Rhizobium borbori* sp. nov.

**Description of *Rhizobium borbori* sp. nov.**

*Rhizobium borbori* (bor’bo.ri. Gr. n. borboros sludge; N.L. gen. n. borbori of sludge).

Cells are Gram-negative rods, motile by one flagellum, that do not form endospores. After 36 h of incubation at 28 °C on LB agar, colonies are circular, convex, semi-translucent and mucilaginous. No anaerobic growth is observed. The optimum temperature for growth is 28 °C; growth occurs at 4 °C but not above 37 °C. The pH range for growth is 5.0–9.0 (optimum pH 6.5–7.2). Sensitive to NaCl; grows in the presence of less than 0.5% NaCl. Utilizes the following carbon sources: L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, D-sorbitol, sucrose, trehalose, turanose, acetic acid, γ-hydroxybutyric acid and propionic acid. The predominant cellular fatty acids are C<sub>18:1</sub>ω7c, C<sub>16:0</sub> and C<sub>19:0</sub> cyclo ω8c.

The type strain, DN316<sup>T</sup> (=CICC 10378<sup>T</sup>=LMG 23925<sup>T</sup>), was isolated from activated sludge of a textile-printing wastewater-treatment plant. The genomic DNA G+C content of strain DN316<sup>T</sup> is 60.9 mol%.

**Acknowledgements**

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**References**


