**Haloimpatiens lingqiaonensis** gen. nov., sp. nov., an anaerobic bacterium isolated from paper-mill wastewater

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An anaerobic bacterium, strain ZC-CMC3\(^\dagger\), was isolated from a wastewater sample in Zhejiang, China. Cells were Gram-stain-positive, peritrichous, non-spore-forming, rod-shaped (0.6–1.2 × 2.9–5.1 \(\mu\)m) and catalase- and oxidase-negative. Strain ZC-CMC3\(^\dagger\) was able to grow at 25–48 °C (optimum 43 °C) and pH 5.5–8.0 (optimum pH 7.0). The NaCl concentration range for growth was 0–3 % (w/v) (optimum 0 %). The major polar lipids of the isolate were diphasphatidylglycerol, phosphatidylglycerol, several phospholipids and glycolipids. Main fermentation products from PYG medium were formate, acetate, lactate and ethanol. Substrates which could be utilized were peptone, tryptone, yeast extract and beef extract. No respiratory quinone was detected. The main fatty acids were C\(_{14}:0\), C\(_{16}:0\), C\(_{16}:1\) \(\text{cis} \ 7\) and C\(_{16}:1\) \(\text{cis} \ 9\). The DNA G+C content was 30.0 mol\%. 16S rRNA gene sequence analysis revealed that the isolate belonged to the family Clostridiaceae. Phylogenetically, the most closely related species were *Oceanirhabdus sediminicola* NH-JN4\(^\dagger\) (92.8 % 16S rRNA gene sequence similarity) and *Clostridium tepidiprofundi* SG 508\(^\dagger\) (92.6 %). On the basis of phylogenetic, chemotaxonomic and phenotypic characteristics, strain ZC-CMC3\(^\dagger\) represents a novel species of a new genus in the family Clostridiaceae, for which the name *Haloimpatiens lingqiaonensis* gen. nov., sp. nov. is proposed. The type strain of the type species is ZC-CMC3\(^\dagger\) (=KCTC 15321\(^\dagger\)=JCM 19210\(^\dagger\)=CCTCC AB 2013104\(^\dagger\)).

According to the second edition of Bergey’s *Manual of Systematic Bacteriology* (Wiegel, 2009), there were 25 genera in the family Clostridiaceae. At the time of writing, according to LPSN (Parte, 2014; http://www.bacterio.net/index.html), several genera have been proposed as new members of the family Clostridiaceae such as *Sporosalibacterium* (Rezgui et al., 2011), *Brassicibacter* (Fang et al., 2012) and *Oceanirhabdus* (Pi et al., 2013) increasing the number of genera to 30. The genus *Clostridium* is the type genus of the family Clostridiaceae (Wiegel, 2009). Taxa in the family Clostridiaceae are generally obligately anaerobic rods and neutrophiles, but several alkaliphilic, alkalithermophilic, moderately halophilic, haloalkaliphilic and slightly acidophilic species have been described. In this paper, we describe an anaerobic strain that cannot tolerate high NaCl concentrations. The strains is proposed to represent a novel species of a new genus belonging to the family Clostridiaceae.

Wastewater samples were collected from a paper mill in Lingqiao town (30° 1′ 59″ N 120° 1′ 09″ E) Zhejiang, China and were stored anaerobically at 4 °C until use. The initial enrichment medium Gs contained (per litre dis-distilled water): 10.0 g NaCl, 1.0 g MgCl\(_2 \cdot \) 6H\(_2\)O, 0.5 g K\(_2\)HPO\(_4\), 0.7 g KH\(_2\)PO\(_4\), 0.025 g FeSO\(_4\) \(\cdot\) 7H\(_2\)O, 0.2 g CaCl\(_2\), 2H\(_2\)O, 1.0 g urea, 5.0 g yeast extract (Difco), 5.0 g tryptone (Difco), 1 ml trace element solution SL-10, 0.4 g L-cysteine and 0.001 g resazurin. To make Gs agar medium, 1.5 % agar was added. To make PYG medium, 10.0 g glucose, 7.0 g yeast extract, 1.0 g tryptone and 5.0 g peptone were added to Gs medium. The wastewater samples were enriched in an anaerobic chamber with Gs medium at 37 °C for 24 h. The enriched samples were then added to Gs agar medium by using the anaerobic agar shake-roll tube technique and cultured at 37 °C until colonies appeared (Hungate, 1969). After several days, one colony, designated ZC-CMC3\(^\dagger\), was picked for further
study. The Hungate roll-tube technique was used to purify the strain at least twice before preservation at −80 °C with 20 % (v/v) glycerol and in dry-freeze ampoules. N₂ was used as the gas phase in all medium.

The temperature range for growth was determined in Gs medium at 4, 15, 19, 25, 34, 40, 43, 45, 48 and 50 °C. The pH range for growth was determined at pH 5.0, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 8.9 as described by Pi et al. (2013). Growth at various NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 %) was determined in modified Gs medium. In modified Gs medium, sodium and chloride ions were removed.

Gram staining was performed using conventional methodology and confirmed using the KOH test (Powers, 1995), Escherichia coli DSM 30083T and Bacillus subtilis DSM 1017T were used as negative and positive controls, respectively. Cell morphology was examined using optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) during stationary growth phase. Oxidase and catalase activities were determined by the methods of Pi et al. (2013). Single carbon source assimilation tests were performed in a basal medium (Gs medium with yeast extract and tryptone removed) supplemented with the following substrates: peptone (10 g l⁻¹), tryptone (10 g l⁻¹), yeast extract (10 g l⁻¹), beef extract (10 g l⁻¹), starch (10 g l⁻¹), glycine (20 mM), pyruvate (25 mM), l-valine (25 mM), D,L-alanine (20 mM), L-proline (10 mM), D,L-alanine (20 mM) plus L-proline (10 mM), L-arginine (25 mM), glucose (25 mM), maltose (25 mM), arabinose (25 mM), fructose (25 mM), xylose (25 mM), cellobiose (25 mM), sucrose (25 mM), sodium formate (20 mM), sodium acetate (20 mM), sodium butyrate (20 mM), sodium fumarate (20 mM), olive oil (10 g l⁻¹), carboxymethyl-cellulose (10 g l⁻¹), filter paper (10 g l⁻¹), chitosan (10 g l⁻¹) and glycerol (20 mM). The major fermentation products in PYG medium after incubation for 48 h were detected by HPLC (Ehrlich et al., 1981). To analyse the reduction of electron acceptors, elemental sulfur (10 g l⁻¹), sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM) and sodium nitrate (20 mM) were added from filter-sterilized solutions to the basal medium (Gs medium lacking l-cysteine and resazurin). The basal medium (Gs medium lacking l-cysteine and resazurin) with one of these electron acceptors but without inoculation were used as chemical controls. The basal medium (Gs medium lacking l-cysteine and resazurin) without electron acceptor but with inoculation was used as blank control. All controls were cultured under same conditions as the experimental groups. Reduction of elemental sulfur, sodium thiosulfate, sodium sulfate and sodium sulfate were tested as described by Pi et al. (2013).

The cells for all chemotaxonomic analyses were incubated in Gs medium at 43 °C for 48 h. Isoprenoid quinones were analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). Fatty acids methyl esters (FAMEs) were obtained as described by Kuykendall et al. (1988). Identification and qualification of the FAMEs were automatically performed by the Sherlock Microbial Identification System with the standard MIS Library Generation Software (MIDI) and the results were matched with the ANAEROBE MOORE 3.90 library. Polar lipids were extracted and then separated on silica gel 60 F254 aluminium-backed thin-layer plates (10 × 10 cm, Merck 5554) and further analysed as described by Minnikin et al. (1984) and Cui et al. (2011).

Genomic DNA was collected using the method described by Marmur & Doty (1962). The 16S rRNA gene was amplified by PCR with the bacterial universal 16S rRNA primer pair 27F (5'-GAGAGTTTGATCCTGCGCTG-3') and 1492R (5'-TACGTTYACCTGGTACGAC-3') (Lane, 1991). PCR products were cloned into the pMD 19-T vector (TaKaRa) for sequencing (Pi et al., 2013). The complete 16S rRNA gene sequence of strain ZC-CMC3T (1476 nt) was identified using the EzTaxon server (http://ezbiocloud.net/eztaxon; Kim et al., 2012). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with the MEGA 5 program package (Tamura et al., 2011). According to the algorithm of Kimura’s two-parameter model (Kimura, 1980) for the neighbour-joining method, evolutionary distances were calculated with the MEGA 5 program package. The DNA G+C content f strain ZC-CMC3T was determined by reversed-phase HPLC according to the method of Mesbah & Whitman (1989).

16S rRNA gene sequence analysis indicated that strain ZC-CMC3T was a member of the phylum Firmicutes, class Clostridia, order Clostridiales, family Clostridiaceae (Collins et al., 1994). The phylogenetic trees reconstructed with all three treeing methods showed that the phylogenetically related species of strain ZC-CMC3T were members of cluster XI of the family Clostridiaceae and strain ZC-CMC3T was closely related to the genera Clostridium and Oceanirhabdus (Fig. 1, and Figs S1 and S2, available in the online Supplementary Material). The results revealed that the isolate was most closely related to Oceanirhabdus sediminicola NH-JN4T and Clostridium tepidiprofundi SG 508T. The 16S rRNA gene sequence similarities between the isolate and O. sediminicola NH-JN4T and C. tepidiprofundi SG 508T were 92.8 % and 92.6 %, respectively. Also, as indicated by the evolutionary distance calculated with Kimura’s two-parameter model in the neighbour-joining tree, strain ZC-CMC3T was more likely to be considered as a novel species of a new genus in the family Clostridiaceae than a novel species in the genus Clostridium or genus Oceanirhabdus. The DNA G+C content of strain ZC-CMC3T (determined by HPLC) was 30.0 mol%.

In Gs agar-shake cultures, white, lens-shaped colonies (0.5–1.0 mm in diameter) of strain ZC-CMC3T appeared after incubation for 48–72 h at 43 °C. Cells were Gram-stain-positive, peritrichous, non-spore-forming and rod-shaped (0.6–1.2 × 2.9–5.1 μm) (Fig. S3). Strain ZC-CMC3T
grew optimally at 43 °C (range 25–48 °C), pH 7.0 (range pH 5.5–8.0) and with 0 % NaCl (range 0–3 %). No growth was observed when the strain was cultured below 25 °C or above 48 °C after incubation for 10 days. No growth was observed in aerobic medium. Detailed results of phenotypic tests are given in Table 1 and in the species description. Strain ZC-CMC3T grew in the absence of electron acceptors by fermentation of peptone, tryptone, yeast extract and beef extract. Sodium sulfite, elemental sulfur, sodium thiosulfate, sodium sulfate, sodium nitrate and sodium nitrite were not reduced and did not stimulate growth with peptone as the electron donor.

The comparison of the physiological and biochemical characteristics of strain ZC-CMC3T, *O. sediminicola* NH-JN4T (Pi et al., 2013) and *C. tepidiprofundus* SG 508T (Slobodkina et al., 2008) is shown in Table 1. Several characteristics were found to discriminate strain ZC-CMC3T from *O. sediminicola* NH-JN4T and *C. tepidiprofundus* SG 508T. The isolation source of strain ZC-CMC3T was wastewater, which is quite distinct from sea sediment (*O. sediminicola* NH-JN4T) and deep-sea hydrothermal vent (*C. tepidiprofundus* SG 508T). Strain ZC-CMC3T showed the optimal growth in 0 % NaCl and could not tolerate NaCl concentrations above 3 %, while the other two strains could not grow in 0 % NaCl but could tolerate NaCl concentrations as high as 6 %. *C. tepidiprofundus* SG 508T could use glucose and maltose for carbohydrate fermentation, but strain ZC-CMC3T and *O. sediminicola* NH-JN4T could not. *O. sediminicola* NH-JN4T could use glycine for amino acid fermentation, but strain ZC-CMC3T and *C. tepidiprofundus* SG 508T showed contrary results. The difference in terms of electron acceptors is that the reference strains *O. sediminicola* NH-JN4T and *C. tepidiprofundus* SG508T were able to reduce sulfur compounds whereas strain ZC-CMC3T was not able to. Additionally, the fermentation products in PYG medium by strain ZC-CMC3T (formate, acetate, lactate and ethanol) showed some differences from *O. sediminicola* NH-JN4T (formate, acetate, butyrate and ethanol) and *C. tepidiprofundus* SG508T (butyrate and ethanol). The detailed fatty acid patterns of strain ZC-CMC3T, *O. sediminicola* NH-JN4T and *C. tepidiprofundus* SG508T are shown in Table S1. The most abundant fatty acid of strain ZC-CMC3T was C16 : 0 (30.9 %) the same as *O. sediminicola* NH-JN4T (20.5 %), but not *C. tepidiprofundus* SG 508T where the major fatty acid was iso-C15 : 0 (38.4 %), which could discriminate strain ZC-CMC3T from the genus *Clostridium*. The main fatty acids of strain ZC-CMC3T were C14 : 0, C16 : 0, C16 : 1 cis 7 and C16 : 1 cis 9. However, the main fatty acids of *O. sediminicola* NH-JN4T and *C. tepidiprofundus* SG 508T were different.

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**Fig. 1.** Neighbour-joining tree using Kimura’s two-parameter model based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain ZC-CMC3T and related species. Numbers at nodes are bootstrap values are based on 1000 replicates; only values >70 % are shown. *Bacillus subtilis* DSM 10T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.
Table 1. Characteristics that differentiate strain ZC-CMC3<sup>T</sup> from Oceanirhabdus sediminicola NH-JN4<sup>T</sup> and Clostridium tepidiprofundi SG508<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Wastewater</td>
<td>Sea sediment</td>
<td>Deep-sea hydrothermal vent</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.6–1.2</td>
<td>0.5–1.2</td>
<td>0.4–0.6</td>
</tr>
<tr>
<td>Width</td>
<td>2.9–5.1</td>
<td>2.2–7.0</td>
<td>2.0–3.0</td>
</tr>
<tr>
<td>Length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>43</td>
<td>34–38</td>
<td>50</td>
</tr>
<tr>
<td>Optimum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>25–48</td>
<td>22–42</td>
<td>22–60</td>
</tr>
<tr>
<td>pH for growth</td>
<td>7.0</td>
<td>6.5–7.0</td>
<td>6.0–6.8</td>
</tr>
<tr>
<td>Optimum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>5.5–8.0</td>
<td>6.0–8.5</td>
<td>4.0–8.5</td>
</tr>
<tr>
<td>NaCl concentration for growth (%)</td>
<td>0</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Optimum</td>
<td>0–3</td>
<td>0.5–6</td>
<td>1–6</td>
</tr>
<tr>
<td>Carbohydrate fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Amino acid fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Reduction of electron acceptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elemental sulfur</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fermentation products in PYG medium</td>
<td>F, A, E, L</td>
<td>F, A, B, E</td>
<td>B, E*</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>30.0</td>
<td>35.8</td>
<td>30.9</td>
</tr>
</tbody>
</table>

*Data from Pi et al. (2013).

from the isolate. For example, iso-C<sub>15</sub>:<sub>0</sub>, C<sub>16</sub>:<sub>0</sub> DMA and unknown 17,103 C<sub>17</sub>:<sub>0</sub> DMA were abundant in O. sediminicola NH-JN4<sup>T</sup>, but were <5.0 % in the isolate. Also, the major contents iso-C<sub>15</sub>:<sub>0</sub>, iso-C<sub>15</sub>:<sub>0</sub> DMA and iso-C<sub>17</sub>:<sub>0</sub> in C. tepidiprofundi SG 508<sup>T</sup> were found in minor amounts in strain ZC-CMC3<sup>T</sup>. These differences in the proportions of main fatty acids could also allow discrimination between strain ZC-CMC3<sup>T</sup> and the genera Clostridium and Oceanirhabdus. The thin-layer chromatograms of polar lipids in strain ZC-CMC3<sup>T</sup> and O. sediminicola NH-JN4<sup>T</sup> are shown in Fig. S4. The major polar lipids of the novel isolate were diphosphatidylglycerol, phosphatidylglycerol, several phospholipids and glycolipids. Meanwhile, some minor contents such as GL4 and PGL1 in strain ZC-CMC3<sup>T</sup> were not found in O. sediminicola NH-JN4<sup>T</sup>.

On the basis of physiological, chemotaxonomic and genotypic characteristics, it is concluded that strain ZC-CMC3<sup>T</sup> represents a novel species of a new genus in the family Clostridiaceae, for which the name Haloimpatiens lingqiaonensis gen. nov., sp. nov. is proposed.

**Description of Haloimpatiens gen. nov.**

Haloimpatiens (Ha.lo.im.pai.tiens. Gr. n. hals, halos salt; L. adj. impatients intolerant; N.L. masc. n. Haloimpatiens salt-intolerant).

Cells are rod-shaped. Chemo-organotrophic and ferments complex proteinaceous compounds. Growth occurs in the absence of NaCl. The main fatty acids are C<sub>14</sub>:<sub>0</sub> and C<sub>16</sub>:<sub>0</sub>. No isoprenoid quinone is detected. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, several phospholipids and glycolipids. Belongs to the family Clostridiaceae.

The type species is Haloimpatiens lingqiaonensis.

**Description of Haloimpatiens lingqiaonensis sp. nov.**

Haloimpatiens lingqiaonensis (ling.qi.a.o.nen’sis. N.L. masc. adj. lingqiaonensis pertaining to the town of Lingqiao in China, where the type strain was isolated).

Cells are Gram-stain-positive, peritrichous, non-spore-forming and 0.6–1.2 × 2.9–5.1 μm. Optimal growth is observed at 43 °C (range 25–48 °C), pH 7.0 (range pH 5.5–8.0) and with 0 % NaCl (range 0–3 %). Catalase- and oxidase-negative. No growth is observed in aerobic medium. Peptone, tryptone, yeast extract and beef extract can support growth, but glycine, pyruvate, L-valine, DL-alanine, L-proline, DL-alanine plus L-proline, L-arginine, glucose, malate, arabinose, fructose, xylose, cellobiose, sucrose, formate, acetate, butyrate, fumarate, olive oil, carboxymethyl-cellulose, filter paper, chitosan and glycerol do not support growth. The major fermentation products from PYG medium are formate, acetate, lactate and ethanol. Sodium sulfite, elemental sulfur, sodium thiosulfate, sodium sulfate, sodium nitrate and sodium nitrite cannot be used as electron acceptors. No respiratory quinone is detected. The major fatty acids are C<sub>14</sub>:<sub>0</sub>, C<sub>16</sub>:<sub>0</sub>, C<sub>16</sub>:<sub>1</sub> cis 7 and C<sub>16</sub>:<sub>1</sub> cis 9.

The type strain is ZC-CMC3<sup>T</sup> (KCTC 15321<sup>T</sup> = JCM 19210<sup>T</sup> = CCTCC AB 2013104<sup>T</sup>), isolated from wastewater of a paper mill in Lingqiao town, Zhejiang, China. The DNA G+C content of the type strain is 30.0 mol%.

**Acknowledgements**

This work was supported by grants from National Natural Science Foundation of China (nos 31260002 and 31470005).
References


