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

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An anaerobic bacterium, strain ZC-CMC3^T, 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 μm) and catalase- and oxidase-negative. Strain ZC-CMC3^T 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 diphosphatidylglycerol, 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 $C_{14:0}$, $C_{16:0}$, $C_{16:1}$ *cis* 7 and $C_{16:1}$ *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^T (92.8 % 16S rRNA gene sequence similarity) and *Clostridium tepidiprofundi* SG 508^T (92.6 %). On the basis of phylogenetic, chemotaxonomic and phenotypic characteristics, strain ZC-CMC3^T 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^T (=KCTC 15321^T=JCM 19210^T=CCTCC AB 2013104^T).

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 distilled water): 10.0 g NaCl, 1.0 g $MgCl_2 . 6H_2O$, 0.5 g K_2HPO_4, 0.7 g KH_2PO_4, 0.025 g $FeSO_4 . 7H_2O$, 0.2 g $CaCl_2 . 2H_2O$, 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^T, was picked for further

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Four supplementary figures and a supplementary table are available with the online Supplementary Material.

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. N_2 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, 37, 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 30083^T and *Bacillus subtilis* DSM 10^T 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), DL-alanine (20 mM), L-proline (10 mM), DL-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 sulfite 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 F_{254} 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′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGAC-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-CMC3^T (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-CMC3^T was determined by reversed-phase HPLC according to the method of Mesbah & Whitman (1989).

16S rRNA gene sequence analysis indicated that strain ZC-CMC3^T 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-CMC3^T were members of cluster XI of the family *Clostridiaceae* and strain ZC-CMC3^T 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-JN4^T and *Clostridium tepidiprofundi* SG 508^T. The 16S rRNA gene sequence similarities between the isolate and *O. sediminicola* NH-JN4^T and *C. tepidiprofundi* SG 508^T 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-CMC3^T 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-CMC3^T (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-CMC3^T 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-CMC3^T

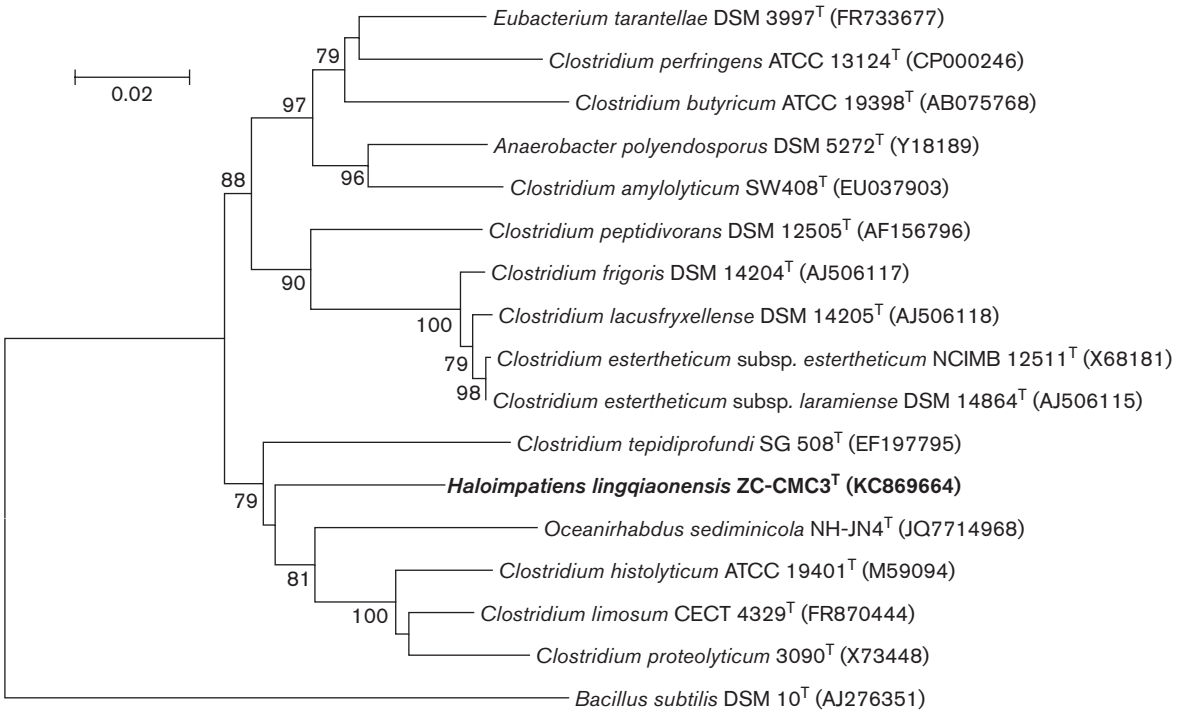

Fig. 1. Neighbour-joining tree using Kimura's two-parameter model based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain ZC-CMC3^T and related species. Numbers at nodes are bootstrap values are based on 1000 replicates; only values >70 % are shown. *Bacillus subtilis* DSM 10^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

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-CMC3^T 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-CMC3^T, *O. sediminicola* NH-JN4^T (Pi *et al.*, 2013) and *C. tepidiprofundi* SG 508^T (Slobodkina *et al.*, 2008) is shown in Table 1. Several characteristics were found to discriminate strain ZC-CMC3^T from *O. sediminicola* NH-JN4^T and *C. tepidiprofundi* SG 508^T. The isolation source of strain ZC-CMC3^T was wastewater, which is quite distinct from sea sediment (*O. sediminicola* NH-JN4^T) and deep-sea hydrothermal vent (*C. tepidiprofundi* SG 508^T). Strain ZC-CMC3^T 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. tepidiprofundi* SG 508^T could use glucose and maltose for carbohydrate fermentation, but strain ZC-CMC3^T and *O. sediminicola* NH-JN4^T could not. *O. sediminicola* NH-JN4^T could use glycine for amino acid fermentation, but strain ZC-CMC3^T and *C. tepidiprofundi* SG 508^T showed contrary results. The difference in terms of electron acceptors is that the reference strains *O. sediminicola* NH-JN4^T and *C. tepidiprofundi* SG508^T were able to reduce sulfur compounds whereas strain ZC-CMC3T was not able to. Additionally, the fermentation products in PYG medium by strain ZC-CMC3^T (formate, acetate, lactate and ethanol) showed some differences from *O. sediminicola* NH-JN4^T (formate, acetate, butyrate and ethanol) and *C. tepidiprofundi* SG 508^T (butyrate and ethanol). The detailed fatty acid patterns of strain ZC-CMC3^T, *O. sediminicola* NH-JN4^T and *C. tepidiprofundi* SG 508^T are shown in Table S1. The most abundant fatty acid of strain ZC-CMC3^T was $C_{16:0}$ (30.9 %) the same as *O. sediminicola* NH-JN4^T (20.5 %), but not *C. tepidiprofundi* SG 508^T where the major fatty acid was iso-$C_{15:0}$ (38.4 %), which could discriminate strain ZC-CMC3^T from the genus *Clostridium*. The main fatty acids of strain ZC-CMC3^T were $C_{14:0}$, $C_{16:0}$, $C_{16:1}$ *cis* 7 and $C_{16:1}$ *cis* 9. However, the main fatty acids of *O. sediminicola* NH-JN4^T and *C. tepidiprofundi* SG 508^T were different

Table 1. Characteristics that differentiate strain ZC-CMC3^T from *Oceanirhabdus sediminicola* NH-JN4^T and *Clostridium tepidiprofundi* SG508^T

Strains: 1, ZC-CMC3^T (data from this study); 2, *O. sediminicola* NH-JN4^T (data from Pi *et al.*, 2013); 3, *C. tepidiprofundi* SG508^T (Slobodkina *et al.*, 2008).+, Positive; −, negative. Fermentation products: F, formate; A, acetate; B, butyrate; E, ethanol; L, lactate.

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

*Data from Pi *et al.* (2013).

from the isolate. For example, iso-$C_{15:0}$, $C_{16:0}$ DMA and unknown 17.103 $C_{17:0}$ DMA were abundant in *O. sediminicola* NH-JN4^T, but were <5.0 % in the isolate. Also, the major contents iso-$C_{15:0}$, iso-$C_{15:0}$ DMA and iso-$C_{17:0}$ in *C. tepidiprofundi* SG 508^T were found in minor amounts in strain ZC-CMC3^T. These differences in the proportions of main fatty acids could also allow discrimination between strain ZC-CMC3^T and the genera *Clostridium* and *Oceanirhabdus*. The thin-layer chromatograms of polar lipids in strain ZC-CMC3^T and *O. sediminicola* NH-JN4^T 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^T were not found in *O. sediminicola* NH-JN4^T.

On the basis of physiological, chemotaxonomic and genotypic characteristics, it is concluded that strain ZC-CMC3^T 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.pa′ti.ens. Gr. n. *hals*, *halos* salt; L. adj. *impatiens* 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 $C_{14:0}$ and $C_{16:0}$. 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, maltose, 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 $C_{14:0}$, $C_{16:0}$, $C_{16:1}$ *cis* 7 and $C_{16:1}$ *cis* 9.

The type strain is ZC-CMC3^T (KCTC 15321^T = JCM 19210^T = CCTCC AB 2013104^T), 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%.

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