**Clostridium aestuarii** sp. nov., from tidal flat sediment

Seil Kim, Hyunyoung Jeong and Jongsik Chun

The western and south-western coasts of the Korean peninsula consist largely of tidal flats, which are unique among other marine sediments in that they are flooded with seawater but periodically exposed. Culture-independent (Kim et al., 2005; Yi & Chun, 2006; Park et al., 2005; Yi & Chun, 2006) studies have shown that the microbial inhabitants of these unique environments are very diverse. A novel anaerobic bacterium, designated strain HY-45-18\(^T\), was isolated from a tidal flat sediment and subjected to a polyphasic taxonomic investigation. Here, we report the taxonomic description of this novel member of the genus *Clostridium*.

Strain HY-45-18\(^T\) was isolated from a sediment sample collected from a tidal flat (37° 35' 31.9" N 126° 27' 24.5" E) on Ganghwa Island, South Korea, using a standard dilution plating method. The isolate was recovered and routinely maintained using marine reinforced clostridial medium (MRCM; Difco) supplemented with 4 % artificial sea salt (Sigma) at 30 °C under anaerobic conditions.

The primers and PCR conditions and the sequencing method for the 16S rRNA gene were as described previously (Chun & Goodfellow, 1995). The resulting sequence of strain HY-45-18\(^T\) was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), and neighbour-joining methods (based on 1000 resamplings). An almost-complete 16S rRNA gene sequence was obtained for strain HY-45-18\(^T\) (1390 bp) and used for an initial BLAST search against the GenBank database. The search result clearly indicated that the tidal flat isolate belonged to cluster I of the order *Clostridiales*, which contains the type species of *Clostridium, Clostridium butyricum*. The closest phylogenetic neighbour of strain HY-45-18\(^T\) was *Clostridium ganghwense* KCTC 5146\(^T\) (96.5 % 16S rRNA gene sequence similarity). Several phenotypic characteristics can be readily used to differentiate the isolate from phylogenetically related clostridia. Therefore, strain HY-45-18\(^T\) represents a novel species of the genus *Clostridium*, for which the name *Clostridium aestuarii* sp. nov. is proposed. The type strain is HY-45-18\(^T\) (= IMSNU 40129\(^T\) = KCTC 5147\(^T\) = JCM 13194\(^T\)).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HY-45-18\(^T\) is D0126679.

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the corresponding level of bootstrap support was as low as 46%.

The Gram reaction was determined using Gram staining and a KOH test (Johnson et al., 1995; Powers, 1995). Morphology was observed for cells grown on MRCM at 30°C by using phase-contrast microscopy and scanning and transmission electron microscopy. The presence of catalase was determined by adding 3% (v/v) H2O2 to cell smears on standard microscope slides. The pH, temperature and sea-salt ranges for growth were determined using MRCM in Hungate tubes. Growth was recorded by measuring the OD600 with a turbidimeter (Biolog). Biochemical tests were performed using the API 20A, API ZYM and API 20NE systems (bioMérieux) in the presence of artificial sea salts and vitamin solution (DSM medium 141) according to the instructions of the manufacturer. Lecithinase activity was assayed using MRCM with egg yolk (Oxoid), according to the instructions of the manufacturer.

To determine the substrates utilized and the end products of fermentation, basal medium (Hernandez-Eugenio et al., 2002) was slightly modified to contain the following (l-1 distilled water): 1 g NH4Cl, 0.3 g K2HPO4, 0.3 g KH2PO4, 30 g sea salt (Sigma), 0.5 g cysteine hydrochloride, 0.3 g Na2S.9H2O, 1 mg resazurin (Sigma), 1 ml trace mineral element solution (DSM medium 318) and 1 ml vitamin solution (DSM medium 141). The final pH was adjusted to 7 with 10 M KOH. After 2 weeks incubation at 30°C, the fermentation end products were analysed using an HPLC apparatus (HP1100; Hewlett Packard) equipped with an Aminex HPX-87H (Bio-Rad) column, a refractory index detector and a diode array detector (210 nm). H2SO4 (0.005 M) was used as the eluent at a flow rate of 0.6 ml min−1. Carbon dioxide and hydrogen were determined using GC (ACME6000GC; Young Lin) equipped with a Porapak Q (Supelco) column and a thermal conductivity detector. Nitrogen was used as the carrier gas, at a flow rate of 20 ml min−1.

The cells of strain HY-45-18T were motile rods with peritrichous flagella. The isolate required sea salts for growth and was unable to grow in the presence of NaCl alone. Detailed morphological, physiological and biochemical characteristics of strain HY-45-18T are given in the species description and Table 1.

It is evident from Table 1 that several phenotypic properties readily separate strain HY-45-18T from other phylogenetically related species, namely C. ganghwense and C. grantii. On the basis of the polyphasic evidence presented here, it is proposed that the tidal flat isolate, strain HY-45-18T, be classified within a novel species of the genus Clostridium, for which the name *Clostridium aestuarii* sp. nov. is proposed.

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**Table 1. Characteristics that differentiate strain HY-45-18T from the type strains of C. ganghwense and C. grantii**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Gram staining</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sea-salt requirement</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mannose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Salicyc</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Fermentation end products</td>
<td>Butrate, propionate, glycerol, ethanol, formate, H2CO2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Acetate, ethanol, glycerol, H2CO2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing relationships among strain HY-45-18T and members of the genus *Clostridium*. Percentages of bootstrap support (based on neighbour-joining analyses of 1000 resampled datasets) are shown at nodes; solid circles indicate that the corresponding nodes (groupings) were also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Clostridium cocleatum* DSM 1551T (Gen-Bank accession no. Y18188) was used as an outgroup (not shown). Bar, 0.1 nucleotide substitutions per position.
Description of Clostridium aestuarii sp. nov.

Clostridium aestuarii (aes.tu.a’ri.i. L. gen. n. aestuarii of the tidal flat).

Cells are strictly anaerobic, chemoheterotrophic, rod-shaped (2–4 × 0.7–0.8 μm) and motile with peritrichous flagella. Spores are oval and terminal. Cells are catalase-negative and lecithinase-negative. Colonies are circular and yellowish on MRCM. Requires 1–10 % (w/v) artificial sea salts (optimum 4%). Does not grow on reinforced clostridial medium containing 0–5 % (w/v) NaCl alone. The optimum temperature for growth is 15–30 °C, with optimum growth at 30 °C. The optimum pH of MRCM for growth is 7.0, and growth occurs between pH 5.5 and 8.5. The KOH reaction and Gram staining are negative. Cannot grow under aerobic conditions. Nitrate is not reduced. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase but not lipase (C14), leucine arylamidase, cystine arylamidase, trypsin, x-chymotrypsin, x-galactosidase, b-galactosidase, b-glucuronidase, a-glucosidase, b-glucosidase, N-acetyl-b-glucosaminidase, x-mannosidase or a-fucosidase. Indole is not produced and urease is absent. Aesculin is hydrolysed but gelatin is not. Glucose, maltose and sucrose are utilized, but arabinose, cellobiose, fructose, galactose, glycerol, lactose, mannitol, mannose, melezitose, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose and xylene are not hydrolysed. The fermentation end products from glucose are butyric acid, propionic acid, glycerol and H2.

The type strain, HY-45-18T (=IMSMNU 40129T = KCTC 5147T = JCM 13194T), was isolated from a tidal flat sediment on Ganghwa Island, South Korea.

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References


