Clostridium aestuarii sp. nov., from tidal flat sediment

Seil Kim, Hyunyoung Jeong and Jongsik Chun

Correspondence

Jongsik Chun
jchun@snu.ac.kr

School of Biological Sciences and Institute of Microbiology, Seoul National University, 56-1 Shillim-dong, Kwanak-gu, Seoul 151-742, Republic of Korea

A strictly anaerobic, halophilic, motile, endospore-forming, rod-shaped bacterium, designated strain HY-45-18^T, was isolated from a sediment sample of a tidal flat in Korea. The isolate produced butyric acid, propionic acid, glycerol and H₂ as fermentation end products from glucose. Strain HY-45-18^T is halophilic as it was unable to grow in the absence of sea salts. A 16S rRNA gene sequence analysis clearly indicated that the tidal flat isolate is a member of 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 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, 2004) and culture-dependent (Choi *et al.*, 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),

maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The alignment and phylogenetic analysis were carried out using the jPHYDIT program (Jeon *et al.*, 2005) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000). The tree topologies obtained were evaluated by using bootstrap analyses (Felsenstein, 1985) of the 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* defined by Collins *et al.* (1994); this cluster contains the type species of the genus *Clostridium*, *Clostridium butyricum*. The newly determined sequence was then aligned manually (on the basis of the secondary structure of the bacterial 16S rRNA) against those of members of cluster I. Phylogenetically, strain HY-45-18^T was closest to *Clostridium ganghwense* KCTC 5146^T (96.5 % 16S rRNA gene sequence similarity). No other species with validly published names showed sequence similarities of 94 % or more. These levels of 16S rRNA gene similarity clearly suggest that our isolate represents a novel species of the genus *Clostridium*. The results of comprehensive phylogenetic analyses are summarized in Fig. 1. Our isolate formed a monophyletic clade with the type strains of *C. ganghwense*, this result being supported by a 99 % bootstrap value and four tree-making algorithms. The type strain of *Clostridium grantii* was associated with this clade, although

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HY-45-18^T is DQ126679.

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing relationships among strain HY-45-18^T 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 1551^T (GenBank accession no. Y18188) was used as an outgroup (not shown). Bar, 0.1 nucleotide substitutions per position.

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

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

Strains: 1, strain HY-45-18^T; 2, *C. ganghwense* KCTC 5146^T (data from Kim *et al.*, 2006); 3, *C. grantii* DSM 8605^T (Mountfort *et al.*, 1994). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3
Gram staining	–	–	+
Sea-salt requirement	+	+	–
Utilization of:			
Sucrose	+	–	–
Mannose	–	+	+
Fructose	–	ND	+
Salicin	–	+	ND
Gelatin	–	+	ND
Cellobiose	–	+	+
Fermentation end products	Butyrate, propionate, glycerol, H ₂	Glycerol, ethanol, CO ₂	Acetate, ethanol, formate, CO ₂

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-45-18^T 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-45-18^T are given in the species description and Table 1.

It is evident from Table 1 that several phenotypic properties readily separate strain HY-45-18^T 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-45-18^T, be classified within a novel species of the genus *Clostridium*, for which the name *Clostridium aestuarii* sp. nov. is proposed.

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 ($2\text{--}4 \times 0.7\text{--}0.8\ \mu\text{m}$) 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 temperature range 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, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -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 xylose are not hydrolysed. The fermentation end products from glucose are butyric acid, propionic acid, glycerol and H₂.

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

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