Clostridium ganghwense sp. nov., isolated from tidal flat sediment

Seil Kim, Hyunyoung Jeong, Sanggoo Kim 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 Gram-negative, strictly anaerobic, halophilic, motile, sporulating and rod-shaped bacterium, designated strain HY-42-06^T, was isolated from tidal flat sediment from Ganghwa Island in South Korea. The isolate produced glycerol, ethanol and CO₂ as fermentation end-products from glucose. Strain HY-42-06^T grew optimally at 35 °C, pH 7.5 and 3 % (w/v) artificial sea salts. No growth was observed in the absence of sea salts. In phylogenetic analyses based on 16S rRNA gene sequence, strain HY-42-06^T showed a distinct phyletic line within the members of cluster I of the order *Clostridiales*. The closest phylogenetic neighbour to strain HY-42-06^T was *Clostridium novyi* ATCC 17861^T (94.91 % 16S rRNA gene sequence similarity). Several phenotypic characters readily differentiate the tidal flat isolate from phylogenetically related clostridia. On the basis of polyphasic evidence, strain HY-42-06^T should be classified as a representative of a novel species, for which the name *Clostridium ganghwense* sp. nov. is proposed. The type strain is HY-42-06^T (=IMSNU 40127^T=KCTC 5146^T=JCM 13193^T).

The prokaryotic inhabitants of tidal flat sediments in Korea have been shown to be very diverse (Kim *et al.*, 2004) and some of them have been isolated and described as novel species (Yi & Chun, 2004; Yi *et al.*, 2003). In this study, we report the taxonomic description of a strictly anaerobic bacterium, designated strain HY-42-06^T.

Strain HY-42-06^T was isolated from a tidal flat sediment sample (37° 35.319' N 126° 27.245' E) from Ganghwa Island, South Korea, using a standard dilution plating method (Jeong *et al.*, 2004). The isolate was recovered and routinely maintained using reinforced *Clostridium* medium (RCM; Difco) supplemented with 4 % artificial sea salts (Sigma) at 25 °C under anaerobic conditions.

Bacterial DNA preparation and PCR amplification and sequencing of 16S rRNA genes were carried out as described previously (Chun & Goodfellow, 1995). The resultant 16S rRNA gene sequence of strain HY-42-06^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, 1993), maximum-parsimony (Fitch,

1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The tree topologies obtained were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. The alignment and phylogenetic analysis were carried out using the jPHYDIT program (available at <http://chunlab.snu.ac.kr/jphydit/>; Jeon *et al.*, 2005) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000).

A nearly complete 16S rRNA gene sequence was obtained for strain HY-42-06^T (1395 bp) and it was used for an initial BLAST search against the GenBank database. The search result clearly indicated that strain HY-42-06^T 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 manually aligned against those of members of cluster I, based on the secondary structure of bacterial 16S rRNA. Phylogenetically, strain HY-42-06^T was most closely related to *Clostridium novyi* ATCC 17861^T (94.91 % 16S rRNA gene sequence similarity), followed by *Clostridium haemolyticum* DSM 5565^T (94.84 %) and *Clostridium homopropionicum* DSM 5847^T (94.81 %). These levels of 16S rRNA gene sequence similarity clearly suggest that strain HY-42-06^T represents a novel species in the genus *Clostridium*. Phylogenetic analysis, based on four different tree-making algorithms, also supported this conclusion, as strain HY-42-06^T formed an independent phyletic line within the *Clostridium* cluster I clade (Fig. 1).

Published online ahead of print on 18 November 2005 as DOI 10.1099/ijs.0.63791-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HY-42-06^T is AY903294.

Scanning and transmission electron micrographs of cells of strain HY-42-06^T are available as supplementary figures in IJSEM Online.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain HY-42-06^T and members of the genus *Clostridium*. Percentages at nodes are levels of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets. *Clostridium cocleatum* DSM 1551^T was used as an out-group. Bar, 0.1 nucleotide substitution per position.

Gram-reaction was determined using Gram-staining and the KOH test (Johnson *et al.*, 1995; Powers, 1995). Morphology was observed for cells grown on RCM at 30 °C using phase-contrast microscopy and scanning and transmission electron microscopy. The presence of catalase was determined by the addition of 3 % (v/v) H₂O₂ to a cell smear on standard microscope slides. The pH, temperature and sea salt ranges for growth were determined using RCM in Hungate tubes. Growth was recorded by measuring OD₆₀₀ using a turbidometer (Biolog). Biochemical tests were performed using the commercially available API 20A system (bioMérieux) according to the manufacturer's instructions.

For the detection of fermentation end-products, 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 salts (Sigma), 0.5 g cysteine hydrochloride, 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, fermentation products were analysed using HPLC (HP1100; Hewlett Packard) equipped with an Aminex HPX-87H (Bio-Rad) column, refractory index detector and diode array detector (210 nm). H₂SO₄ (0.005 M) was used as eluent at a flow rate of 0.6 ml min⁻¹. Carbon dioxide was analysed using GC (ACME6000GC; Young-lin) equipped with a Porapak Q (Supelco) column and thermal conductivity detector. Helium was used as the carrier gas at a flow rate of 20 ml min⁻¹.

Cells were motile rods with peritrichous flagella (see Supplementary Figs S1 and S2 in IJSEM Online). 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-42-06^T are given in the species description and Table 1.

It is evident from Table 1 that several phenotypic characters readily separate strain HY-42-06^T from other phylogenetically related species, namely *C. novyi*, *C. haemolyticum* and *C. homopropionicum*. On the basis of the polyphasic evidence presented here, isolate HY-42-06^T represents a novel species in the genus *Clostridium* for which the name *Clostridium ganghwense* sp. nov. is proposed.

Table 1. Characteristics that differentiate strain HY-42-06^T from phylogenetically related species

Strains: 1, Strain HY-42-06^T; 2, *Clostridium novyi* ATCC 17861^T; 3, *Clostridium haemolyticum* DSM 5565^T; 4, *Clostridium homopropionicum* DSM 5847^T. Data are from this and earlier studies (Breed *et al.*, 1957; Dorner & Schink, 1990). +, Positive reaction; -, negative reaction; v, variable; ND, no data available.

Characteristic	1	2	3	4
Gram-staining	–	+	+	–
Sea salts requirement	+	–	–	–
Indole production	+	–	+	ND
Utilization of:				
Glucose	+	+	+	–
Glycerol	–	v	+	–
Maltose	+	v	–	ND
Salicin	+	–	–	ND

Description of *Clostridium ganghwense* sp. nov.

Clostridium ganghwense (gang.hwen'se. N.L. neut. adj. *ganghwense* named after Ganghwa Island in South Korea, the geographical origin of the type strain).

Strictly anaerobic, chemoheterotrophic, rod-shaped ($4\text{--}8\text{ }\mu\text{m} \times 0.7\text{--}0.8\text{ }\mu\text{m}$) and motile with peritrichous flagella. Cells are catalase-negative. Colonies are circular and yellowish on RCM. Requires 1–9 % (w/v) artificial sea salts (optimum 3 %). Does not grow on RCM containing 0–5 % (w/v) NaCl alone. The temperature range for growth is 15–40 °C, optimum growth temperature is 35 °C. Optimum pH of RCM for growth is 7.5 and growth occurs between pH 5.5 and 10.0. The KOH reaction and Gram-staining are negative. Indole is produced and urease is absent. Gelatin and aesculin are hydrolysed. Glucose, maltose, salicin, cellobiose and mannose are utilized, but mannitol, lactose, sucrose, xylose, arabinose, glycerol, melezitose, raffinose, sorbitol, rhamnose and trehalose are not utilized. The fermentation end-products from glucose are glycerol, ethanol and CO₂.

The type strain, HY-42-06^T (=IMSNU 40127^T=KCTC 5146^T=JCM 13193^T), was isolated from tidal flat sediment of Ganghwa Island in South Korea.

Acknowledgements

This work was supported, in part, by the Korea Ministry of Science and Technology under National Research Laboratory Program (M10500000110-05J0000-11010), 21C Frontier Microbial Genomics and Applications Center Program (MG05-0101-2-0) and Korea Institute of Science and Technology.

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