Clostridium jejuense sp. nov., isolated from soil

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A strictly anaerobic, mesophilic, endospore-forming bacterium, designated strain HY-35-12^T, was isolated from a soil sample in Jeju, Korea. Cells of this isolate were Gram-positive, motile rods that formed oval to spherical terminal spores. Strain HY-35-12^T grew optimally at 30 °C, pH 7.0 and 0–0.5 % (w/v) NaCl. The isolate produced pyruvate, lactate, acetate, formate and hydrogen as fermentation end products from glucose. The G + C content of DNA of the isolate was 41 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism formed a monophyletic clade with *Clostridium xylanovorans* and *Clostridium aminovalericum* in cluster XIVa of the genus *Clostridium*. The closest phylogenetic neighbour was *C. xylanovorans*, with 96.65 % 16S rRNA gene sequence similarity. Several physiological and chemotaxonomic properties were identified that enable strain HY-35-12^T to be distinguished from phylogenetically related clostridia. On the basis of polyphasic characteristics, it is proposed that strain HY-35-12^T (=IMSNU 40003^T=KCTC 5026^T=DSM 15929^T) represents a novel species, *Clostridium jejuense* sp. nov.

The genus *Clostridium* encompasses Gram-positive, endospore-forming, strictly anaerobic bacteria. At present, the genus comprises over 140 phylogenetically heterogeneous species. Clostridia have been routinely found in soil and are implicated as major players in the decomposition of various organic compounds (Hippe *et al.*, 1992). In this study, a polyphasic characterization of a strictly anaerobic, endospore-forming strain, isolated from soil, is reported. It is proposed that this strain, HY-35-12^T, represents a novel species, *Clostridium jejuense* sp. nov.

Strain HY-35-12^T was isolated from a soil sample collected in Jeju, Korea (33° 21' 36.3" N 126° 52' 05.8" E), using the standard dilution plating method. The isolate was recovered from a reinforced clostridial (RC; Difco) agar plate at 30 °C under anaerobic conditions (N₂/CO₂/H₂, 90:5:5, by vol.). *Clostridium xylanovorans* DSM 12503^T and *Clostridium aminovalericum* DSM 1283^T were used as reference strains. Test strains were routinely cultured on RC plates and maintained as glycerol suspensions (20 %, w/v) at –80 °C.

Bacterial DNA preparation and PCR amplification and sequencing of 16S rRNA genes were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequences were aligned manually against sequences obtained from GenBank. Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The resultant tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The alignment and phylogenetic analyses were carried out using the programs PHYDIT (available at <http://plaza.snu.ac.kr/~jchun/phydit/>) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000).

A nearly complete 16S rRNA gene sequence (1435 bp) of the isolate was obtained and used for the initial BLAST search against GenBank and preliminary neighbour-joining analysis (available as supplementary material in IJSEM Online). Strain HY-35-12^T was most closely related to members of cluster XIVa in the genus *Clostridium* (Collins *et al.*, 1994). The closest phylogenetic neighbour was *C. xylanovorans*, with 96.65 % 16S rRNA gene sequence similarity, followed by *C. aminovalericum*, with 93.54 % 16S rRNA gene sequence similarity. To elucidate the phylogenetic relationship between our isolate and other members of *Clostridium* cluster XIVa, phylogenetic trees

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The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain HY-35-12^T is AY494606.

A phylogenetic tree based on preliminary neighbour-joining analysis is available as supplementary material in IJSEM Online.

Fig. 1. Neighbour-joining tree of strain HY-35-12^T and related taxa within cluster XIVa in the genus *Clostridium* based on 16S rRNA gene sequences. Numbers at nodes are levels of bootstrap support (percentages) based on neighbour-joining analyses of 1000 resampled datasets. *Clostridium perfringens* ATCC 13124^T (M59103) was used as an outgroup (not shown). Bar, 0.1 nt substitution per position.

were constructed using four different tree-making algorithms. The neighbour-joining tree (Fig. 1) showed that strain HY-35-12^T clustered with the type strain of *C. xylanovorans* with 100% bootstrap support. These two then formed a monophyletic clade with *C. aminovalericum* with 99% bootstrap support. This relationship was confirmed by all other tree-inferring methods used in this study. On the basis of 16S rRNA gene similarity data and phylogenetic analysis, it is clear that our isolate belongs to a novel genomic species in cluster XIVa of the genus *Clostridium*.

Gram-staining and the KOH test were carried out according to Johnson *et al.* (1995) and Powers (1995), respectively. Morphology of cells grown on RC agar plates at 30 °C for 72 h was observed using light microscopy and SEM. Motility of strain HY-35-12^T was checked in a Hungate tube (Bellco) containing RC semi-solid medium and sulfite indole motility (Difco) semi-solid medium with 0.5% agar. Flagella type was determined using TEM. Endospores were observed using light microscopy; background staining was done with 10% (w/v) nigrosine (Sigma). The presence of catalase was determined by addition of 10% (v/v) H₂O₂ to a cell smear on standard microscope slides. The pH, temperature and NaCl ranges for growth were determined using modified tryptic soy broth (mTSB) medium (l⁻¹: tryptone, 15 g; soytone, 5 g; NaCl 5 g; cysteine hydrochloride, 0.5 g; resazurin, 0.001 g) in Hungate tubes. Growth was recorded by measuring OD₆₀₀ in Hungate tubes using a turbidometer (Biolog).

Substrate utilization patterns of test strains were determined at 30 °C and an initial pH of 7.0 according to the procedure of Mechichi *et al.* (1999). The results were recorded after 2 weeks incubation. Various biochemical tests were carried out using the API 20A system (bioMérieux) according to the manufacturer's instructions.

For detection of fermentation end products, basal medium was prepared based on that of Widdel & Pfennig (1981), but slightly modified. It contained the following (l⁻¹ distilled water): NH₄Cl, 0.535 g; KH₂PO₄, 0.136 g; MgCl₂·6H₂O, 0.204 g; CaCl₂·2H₂O, 0.147 g; trace element solution (DSMZ medium 318), 1 ml; vitamin solution (DSMZ medium 141), 1 ml; resazurin solution, 1 ml; NaHCO₃, 2.52 g; Na₂S·9H₂O, 0.3 g; and cysteine hydrochloride, 0.3 g (pH 7.0). All cultivations were carried out at 30 °C in 50 ml serum vials containing 20 ml medium under an atmosphere of N₂/CO₂ (80:20, v/v). Glucose solution was autoclaved and added to the medium prior to cultivation using a sterilized syringe to give a final concentration of 20 mM. Hydrogen was analysed by GC (Shimadzu GC-8AIT) equipped with Unibeads C and a thermal conductivity detector. Argon was used as a carrier at a flow rate of 27 ml min⁻¹. The column and detector temperatures were kept at 60 and 110 °C, respectively. Carboxylic acids were determined by HPLC (Shimadzu LC-6A) equipped with an SCR-101H (Shimadzu) column and a model SPD-6A UV detector. The column was operated at 40 °C and 60% HClO₄ was used as eluent at a flow rate of 1.8 ml min⁻¹.

For determination of G+C content, DNA was extracted and purified according to a method described previously (Wolff & Gemmill, 1997) and the G+C content of strain HY-35-12^T was determined by HPLC of deoxyribonucleosides as described by Mesbah *et al.* (1989) using a reverse-phase column (Supelcosil LC-18-S; Supelco).

Cells of strain HY-35-12^T in exponential growth phase occurred singly and were straight to slightly curved rods. Endospore formation was observed in a liquid culture of mTSB medium containing fructose. The cells swelled to form slightly oval to almost spherical terminal spores from the mid-exponential phase of growth. Cells were motile in semi-solid medium and TEM revealed that the organism had peritrichous flagella. Gram-staining and the KOH reaction indicated a Gram-positive cell wall. No growth of strain HY-35-12^T occurred under aerobic conditions. Cells grew at 10–40 °C, pH 5.5–9.5 and 0–1.5% (w/v) NaCl; optimal growth was observed at 30 °C, pH 7.0 and 0–0.5% (w/v) NaCl.

The detailed results of biochemical, substrate utilization, DNA G+C content and fermentation end product analyses are given in Table 1 and the species description. It is evident from Table 1 that there are several phenotypic characters that readily separate strain HY-35-12^T from phylogenetically related species, namely *C. aminovalericum* and *C. xylanovorans*. *C. aminovalericum* has a significantly

Table 1. Characteristics that differentiate strain HY-35-12^T from phylogenetically related *Clostridium* species

Strains: 1, strain HY-35-12^T; 2, *C. xylanovorans* DSM 12503^T; 3, *C. aminovalericum* DSM 1283^T. All strains were Gram-positive motile rods, hydrolysed aesculin and utilized D-cellobiose, D-glucose, D-maltose, D-mannose, sucrose, D-trehalose, D-fructose, D-galactose and D-ribose. All strains were negative for indole production and urease and protease activities. +, Positive; –, negative reaction; W, weakly positive.

Characteristic	1	2	3
G + C content (mol%)	41	40*	33†
Optimum growth temperature (°C)	30	37*	37†
Presence of catalase	–	+	–
Utilization of:‡			
L-Arabinose	+	–	W
Lactose	+	+	–
D-Raffinose	+	+	–
D-Sorbitol	–	–	W
D-Xylose	+	–	+
Cellulose	W	–	+
D-Mannitol	–	+	+
Yeast extract (0.2%)	–	–	W

*Results from Mechichi *et al.* (1999).

†Results from Hardman & Stadtman (1960).

‡Results obtained using the method of Mechichi *et al.* (1999).

lower DNA G + C content (33 mol%) than strain HY-35-12^T and *C. xylanovorans*. On the basis of the polyphasic evidence presented here, it is proposed that the soil isolate HY-35-12^T be classified as a representative of a novel *Clostridium* species, *Clostridium jejuense* sp. nov.

Description of *Clostridium jejuense* sp. nov.

Clostridium jejuense (je.ju.en'se. N.L. neut. adj. *jejuense* pertaining to Jeju Island, Korea, geographical origin of the type strain of the species).

Gram-positive, strictly anaerobic, mesophilic bacterium. Cells are straight to slightly curved rods, 1.8–4.5 µm long and 0.5 µm wide, motile by means of peritrichous flagella. Forms slightly oval to almost spherical terminal endospores. Colonies on RC agar plates are 1.0–2.5 mm in diameter, circular, entire, flat, translucent to opaque, greyish and smooth after 72 h. Cells grow at 10–40 °C, pH 5.5–9.5 and 0–1.5% (w/v) NaCl; optimum growth occurs at 30 °C, pH 7.0 and 0–0.5% (w/v) NaCl. The doubling time under optimum conditions is 10.5 h. Catalase is absent and indole is not produced. Aesculin is hydrolysed, but not gelatin or urea. Utilizes D-cellobiose, D-glucose, lactose, D-maltose, D-mannose, sucrose, D-trehalose, D-fructose, D-galactose, D-ribose, L-arabinose, D-raffinose, D-xylose and cellulose, but not D-sorbitol, D-mannitol, peptone (0.1%, w/v) or yeast extract (0.2%, w/v). Fermentation end products from glucose include pyruvate, lactate, acetate,

formate and H₂. The G + C content of the DNA is 41 mol%. Detailed biochemical characteristics are given in Table 1. Phylogenetically, the organism belongs to cluster XIVa of the genus *Clostridium sensu* Collins *et al.* (1994).

The type strain is HY-35-12^T (=IMSNU 40003^T = KCTC 5026^T = DSM 15929^T), isolated from soil in Jeju, Korea.

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