Halobacillus faecis sp. nov., a spore-forming bacterium isolated from a mangrove area on Ishigaki Island, Japan

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A Gram-positive, spore-forming, rod-shaped halophilic bacterial strain, IGA7-4^T, was isolated from a mangrove area on Ishigaki Island (Japan), and was characterized taxonomically using a polyphasic approach. Strain IGA7-4^T was strictly aerobic and non-motile and formed central endospores. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain IGA7-4^T is affiliated to the genus *Halobacillus*, and exhibits sequence similarities of 99.6–98.0% to the type strains of *Halobacillus* species. Levels of DNA–DNA relatedness between strain IGA7-4^T and the type strains of *Halobacillus* species were 9.5–46.6%. The DNA G + C content of strain IGA7-4^T was 46.5 mol%. The cell-wall peptidoglycan type (Orn–Asp), major cellular fatty acids (anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}) and quinone type (MK-7) of the isolate support its affiliation to the genus *Halobacillus*. On the basis of phylogenetic analysis, phenotypic characteristics and chemotaxonomic data, the isolate represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus faecis* sp. nov. is proposed. The type strain is IGA7-4^T (=MBIC08268^T=IAM 15427^T=KCTC 13121^T).

The genus *Halobacillus* was first described by Spring *et al.* in 1996 and, at the time of writing, comprised nine recognized species: *Halobacillus halophilus*, *Halobacillus litoralis* and *Halobacillus trueperi* (Spring *et al.*, 1996), *Halobacillus salinus* (Yoon *et al.*, 2003), *Halobacillus karajensis* (Amoozegar *et al.*, 2003), *Halobacillus locisalis* (Yoon *et al.*, 2004), *Halobacillus dabanensis* and *Halobacillus aidingensis* (Liu *et al.*, 2005) and *Halobacillus yeomjeoni* (Yoon *et al.*, 2005). Members of the genus *Halobacillus* have a cell-wall peptidoglycan type based on L-Orn–D-Asp, which clearly differentiates them from other related genera. In this study, we report a strain, IGA7-4^T, isolated from a mangrove area (Ishigaki Island, Japan) in the course of environmental investigation. On the basis of phenotypic characteristics, chemotaxonomic data and phylogenetic analysis of the 16S rRNA gene sequence, the isolate represents a novel species of *Halobacillus*.

Strain IGA7-4^T was isolated from sediment collected from a mangrove area of the estuary of the Nagura River in Ishigaki Island, Okinawa, Japan in 2004. Orange-coloured

colonies appeared after incubation for 1 week at 25 °C on 1/10-diluted marine agar [3.74 g Difco marine broth 2216 (BD), 750 ml filtered seawater, 250 ml distilled water and 15 g agar]. Isolate IGA7-4^T was routinely cultivated on marine agar at 30 °C. Cell morphology and motility were examined by using phase-contrast microscopy (BX60 microscope; Olympus). Growth under anaerobic conditions was determined after incubation for 1 week in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.). Catalase activity was tested by adding a drop of 3% H₂O₂ to a single colony and was recorded as positive when development of bubbles was observed. Oxidase activity was determined by using cytochrome oxidase paper (Nissui Pharmaceutical Co., Ltd). API 20E, API 50 CH and API ZYM microtest galleries (bioMérieux) were used to determine physiological and biochemical characteristics. The API 20E and API 50 CH strips were incubated for 48 h at 37 °C and the API ZYM strip was recorded after incubation for 4 h at 30 °C.

The isolate was Gram-positive, strictly aerobic, non-motile and rod-shaped. The morphological and physiological characteristics are provided in the species description. The isolate showed differences from other species of *Halobacillus* in some physiological properties (Table 1).

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain IGA7-4^T is AB243865.

Table 1. Differential characteristics of *Halobacillus* species

Taxa: 1, strain IGA7-4^T (*H. faecis* sp. nov.); 2, *H. litoralis* (data from Spring *et al.*, 1996); 3, *H. halophilus* (Claus *et al.*, 1983; Spring *et al.*, 1996); 4, *H. trueperi* (Spring *et al.*, 1996); 5, *H. salinus* (Yoon *et al.*, 2003); 6, *H. karajensis* (Amoozegar *et al.*, 2003); 7, *H. locisalis* (Yoon *et al.*, 2004); 8, *H. dabanensis* (Liu *et al.*, 2005); 9, *H. aidingensis* (Liu *et al.*, 2005); 10, *H. yeomjeoni* (Yoon *et al.*, 2005). +, Positive; –, negative; w, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell morphology	Rods	Rods	Cocci or oval	Rods	Rods	Rods	Rods	Rods	Rods	Rods or long filamentous rods
Motility	–	+	+	+	+	–	+	+	+	+
Spore shape*	E, S	E, S	S	E, S	E	E, S	E	E	E	E
Spore position†	C	C, ST	C, L	C, ST	C, ST	C, ST	C, ST	C, ST	C, ST	C, ST
Colony colour	Orange	Orange	Orange	Orange	Pale orange–yellow	Cream or white	Light orange–yellow	Cream	Orange	Light yellow
Temperature range for growth (°C)	15–45	15–43	10–40	10–44	10–45	10–49	10–42	15–50	15–40	15–48
pH range for growth	5.5–9.0	6.0–9.5	7.0–9.0	6.0–9.5	ND	6.0–9.6	5.0–9.5	5.0–11.0	6.0–10.0	ND
NaCl range for growth (% w/v)	0–24	0.5–25	2–20	0.5–30	0–23	1–24	0–23	0.5–25	0.5–20	0.5–21
Acid production from:										
D-Fructose	+	+	–	+	+	+	+	+	+	–
D-Galactose	+	–	–	+	w	–	–	–	–	–
Maltose	+	+	–	+	+	+	–	+	+	+
Sucrose	+	+	–	+	+	–	+	+	+	+
D-Xylose	–	+	–	–	–	–	–	+	–	–
D-Glucose	+	+	–	+	+	+	+	+	+	+
D-Mannitol	+	+	–	–	+	+	–	+	+	+
Trehalose	+	+	–	+	+	ND	+	+	+	+
DNA G + C content (mol%)	46.5	42.0	40.1–40.9	43.0	45.0	41.3	44.0	41.4	42.2	42.9

*E, Ellipsoidal; S, spherical.

†C, Centre; L, lateral; ST, subterminal.

Based on partial 16S rRNA gene sequence analyses (Goto *et al.*, 2000, 2002), strain IGA7-4^T was grouped in the *Halobacillus* cluster. However, it was found to be distinct from previously described species of the genus. The 16S rRNA gene sequence was determined using a 16S rRNA Gene kit following the protocols of the manufacturer (Applied Biosystems). The 16S rRNA gene sequence of strain IGA7-4^T was compared with sequences obtained from GenBank (National Centre for Biotechnology Information). The sequences were aligned using the CLUSTAL_X software package (Thompson *et al.*, 1997), and the evolutionary distances and K_{nuc} values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not taken into consideration when 1416 bases of the 16S rRNA gene nucleotides were compared. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of the phylogenetic tree was evaluated using the bootstrap

resampling method of Felsenstein (1985), with 1000 replicates. Similarity values were calculated using MEGA3 (Kumar *et al.*, 2004).

An almost-complete 16S rRNA gene sequence of strain IGA7-4^T was determined and subjected to comparative analysis. Strain IGA7-4^T showed sequence similarities of 99.6–98.0% to the type strains of *Halobacillus* species. On the other hand, 16S rRNA sequence similarities of less than 97.0% were obtained with species of other related genera. The phylogenetic tree indicated that strain IGA7-4^T was closely related to the genus *Halobacillus* (Fig. 1).

Genomic DNA was prepared according to the method of Marmur (1961). DNA–DNA hybridization was performed by the photobiotin-labelling method of Ezaki *et al.* (1989) using a multi-well plate reader (CytoFluoR; PerSeptive Biosystems). The level of DNA–DNA relatedness between strain IGA7-4^T and the type strains of *H. trueperi*, *H.*

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain IGA7-4^T and other related taxa. Numbers at nodes are percentage bootstrap values based on 1000 replications; only values greater than 70% are shown. Bar, 0.01 substitutions per nucleotide position.

aidingensis, *H. dabanensis*, *H. karajensis*, *H. litoralis*, *H. localis*, *H. yeomjeoni*, *H. halophilus* and *H. salinus* were 46.6, 39.5, 17.6, 14.5, 14.2, 12.5, 11.4, 10.3 and 9.5%, respectively. These levels of DNA–DNA relatedness were less than the threshold value of 70% suggested for separate species delineation by Wayne *et al.* (1987).

The G+C content of the total DNA was measured by HPLC according to the method described by Mesbah *et al.* (1989). The DNA G+C content of strain IGA7-4^T was 46.5 mol%, which is slightly higher than those reported for other species of the genus *Halobacillus* (Table 1).

Cellular fatty acids of strain IGA7-4^T grown on marine agar for 3 days at 25 °C were prepared, separated and identified using the Microbial Identification System (MIDI, Inc.). The fatty acids (>1% of the total fatty acids) of strain IGA7-4^T were anteiso-C_{15:0} (50.0%), anteiso-C_{17:0} (15.0%), iso-C_{15:0} (13.8%), iso-C_{16:0} (9.8%), iso-C_{17:0} (2.9%), iso-C_{14:0} (2.5%), C_{16:0} (2.1%), C_{16:1}ω7c alcohol (1.9%) and C_{15:0} (1.2%). This fatty acid profile is similar to those of other *Halobacillus* species (Yoon *et al.*, 2003, 2004; Amoozegar *et al.*, 2003; Liu *et al.*, 2005; Yoon *et al.*, 2005).

Analysis of cell-wall peptidoglycan of strain IGA7-4^T was carried out according to the methods described by Schleifer & Kandler (1972). Strain IGA7-4^T possessed a cell-wall type of Orn–Asp. Respiratory quinone analysis of strain IGA7-4^T was performed according to the method of Collins & Jones (1981). The major isoprenoid quinone was MK-7.

On the basis of phenotypic, chemotaxonomic and phylogenetic data, we conclude that strain IGA7-4^T belongs to a novel species of the genus *Halobacillus*, for which the name *Halobacillus faecis* sp. nov. is proposed.

Description of *Halobacillus faecis* sp. nov.

Halobacillus faecis (fae'cis. L. gen. n. *faecis* of dredge, sediment, from which the organism was isolated).

Cells are Gram-positive, rods (1.6–1.8 × 2.4–3.4 μm) and non-motile. Ellipsoidal and spherical spores are formed in

a central position. Colonies on marine agar are circular, convex and orange. Growth occurs at 15–45 °C and pH 5.5–9.0. NaCl is not required for growth but can be tolerated up to 15% NaCl (w/v). Tests for catalase, gelatinase and β-galactosidase activities are positive. H₂S, acetoin and indole are not produced. Nitrate and nitrite are not reduced. Tests for oxidase, urease and tryptophan deaminase activities and citrate utilization are negative. Tests for arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are negative. Acid is produced from glycerol, ribose, galactose, glucose, fructose, mannitol, *N*-acetylglucosamine, aesculin, maltose, sucrose, trehalose, starch and glycogen, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. In assays using the API ZYM system, alkaline phosphatase, esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase and α-glucosidase are positive, but esterase (C4), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The cell wall contains peptidoglycan of the Orn–Asp type. The major isoprenoid quinone is MK-7. Major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}. The DNA G+C content of the type strain is 46.5 mol%.

The type strain, IGA7-4^T (=MBIC08268^T=IAM 15427^T=KCTC 13121^T), was isolated from field soil in a mangrove area in Ishigaki Island, Japan.

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