NOTE

***Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan**

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Two Gram-positive, endospore-forming, alkaliphilic bacteria were isolated from water samples obtained from the Okuhida hot spa area of Japan. The unknown bacteria were characterized using phenotypic and molecular taxonomic methods. On the basis of phylogenetic evidence and phenotypic distinctiveness, a new species, *Bacillus okuhidensis* sp. nov., is proposed. The type strain of *Bacillus okuhidensis* is GTC 854^T (= JCM 10945^T = DSM 13666^T).

Keywords: *Bacillus okuhidensis*, 16S rRNA, alkaliphilic, DNA–DNA hybridization

Alkaliphilic micro-organisms, species of the genus *Bacillus* in particular, are of considerable biotechnological importance because of their ability to produce some extracellular alkaline enzymes such as protease (Horikoshi, 1971), pectinase (Horikoshi, 1972) and amylases (Boyer & Ingle, 1972) that are resistant to high-pH or high-temperature conditions (Hamamoto & Horikoshi, 1992; Nielsen *et al.*, 1994). These unusual enzymes can be used in industrial and other processes (Nielsen *et al.*, 1995; Yamagata, 1993).

Since Vedder (1934) first isolated the aerobic, endospore-forming, obligate alkaliphilic micro-organism *Bacillus alcalophilus*, many strains of obligate or facultative alkaliphiles have been isolated for industrial applications and physiological studies (Horikoshi, 1991; Nielsen *et al.*, 1995; Yumoto *et al.*, 1998). Gordon & Hyde (1982) grouped these alkaliphilic strains into the *Bacillus firmus* and *Bacillus lentus* groups and the *B. firmus*–*B. lentus* complex. These strains and other isolates were redefined by Fritze *et al.* (1990). On the basis of the phenotypic characteristics correlated with distinct mol% G+C contents, they concluded that these alkaliphilic *Bacillus* strains should be classified as various species according to the low DNA–DNA similarity values between strains in the same group. In the present investigation, two alkaliphilic bacteria isolated from water samples were subjected to a polyphasic study designed to establish their taxonomic positions. Genotypic and phenotypic

data show that the isolated strains represent a new species of *Bacillus*, for which we propose the name *Bacillus okuhidensis*.

Two hot-water samples were collected from the Okuhida hot spa area in Japan. The temperatures and pH values of the sources of the two isolates were 59 °C/pH 6.4 and 51 °C/pH 8.1. Strains were deposited with the Gifu Type Culture collection (GTC) and designated as GTC 854^T and GTC 855. The primary culture medium was heart infusion broth (Difco). To determine the optimal pH for growth, isolates were cultured at 45 °C by shaking in an incubator (Eyela Shaker III; Tokyo Rikakikai) at speed 5. The pH of the media was adjusted with 1 M solutions of K₃PO₄ and HCl. Growth was monitored by determining the turbidity at 650 nm with a spectrophotometer (Ultraspec 3000; Pharmacia) according to the method described by Yamagata (1993). Analysis of the effect of NaCl on the growth of cells was carried out in heart infusion broth, adjusted to pH 10.5, and the culture was shaken at 45 °C as described above. Both isolates showed growth at the lowest temperature, 30 °C, and showed optimal growth at temperatures between 45 and 50 °C in heart infusion medium with the pH adjusted to 10.5. Strain GTC 854^T grew at up to 60 °C, but the maximum temperature for growth of strain GTC 855 was 55 °C. Both strains grew well at pH values ranging from 6.0 to 11.0 at 45 °C. Optimal growth was observed at pH 10.5. They also grew in the presence of up to 10% NaCl (w/v) at pH 10.5 and 45 °C (Table 1).

Organisms were observed through a phase-contrast light microscope (Optiphot XF-NT; Nikon) to determine the Gram-staining and endospore-forming characteristics. Detailed morphological studies were

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The DDBJ/GenBank accession numbers for the 16S rRNA gene sequences of GTC 854^T and GTC 855 are AB047684 and AB047685, respectively.

Table 1. Phenotypic characteristics of *B. okuhidensis* and other alkaliphilic *Bacillus* species

Species: 1, *B. okuhidensis*; 2, *B. halodurans*; 3, *B. alcalophilus*; 4, *B. pseudocalophilus*; 5, *B. pseudofirmus*; 6, *B. cohnii*; 7, *B. clausii*; 8, *B. horikoshii*; 9, *B. clarkii*; 10, *B. agaradhaerens*; 11, *B. gibsonii*; 12, *B. halmapalus*. +, Positive; –, negative; v, variable; ND, no data. All species grow in 5% NaCl and hydrolysed gelatin and starch. Data for species 2–12 are from Nielsen *et al.* (1995) and Yumoto *et al.* (1998).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth at:												
20 °C	–	+	+	+	+	v	+	+	+	+	+	+
40 °C	+	+	+	+	+	+	+	+	+	+	–	+
45 °C	+	+	–	–	+	+	+	–	+	+	–	–
50 °C	+	+	–	–	–	–	+	–	–	–	–	–
55 °C	+	+	–	–	–	–	–	–	–	–	–	–
60 °C	v*	–	–	–	–	–	–	–	–	–	–	–
pH												
pH 6	+	–	–	–	–	–	–	–	–	–	–	–
pH 7	+	v	–	–	v	v	+	+	–	–	+	+
pH 9	+	+	+	+	+	+	–	–	+	+	–	–
pH 10	+	v	v	+	v	–	–	–	+	+	–	–
pH 11	+	–	–	–	v	–	–	–	+	+	–	–
Growth in NaCl at:												
8 %	+	+	+	+	+	v	+	+	+	+	+	–
10 %	+	+	–	+	+	–	v	–	+	+	v	–
12 %	–	+	–	–	+	–	–	–	+	+	v	–
13 %	–	v	–	–	+	–	–	–	+	+	–	–
Reduction of nitrate	+	v	v	–	–	+	+	–	+	+	v	–
Deamination of phenylalanine	–	–	–	–	+	–	–	–	–	–	–	–
Utilization of:												
L-Arabinose	+	v	+	+	v	ND	+	–	ND	+	+	–
D-Xylose	+	+	v	+	v	ND	+	v	ND	+	v	v
Salicin	+	+	+	+	v	ND	+	–	ND	+	+	v
Glycogen	+	+	+	+	v	ND	+	+	ND	+	–	+
Inositol	+	+	v	–	v	ND	v	–	ND	–	–	–
Melibiose	+	+	+	–	–	ND	+	–	ND	+	+	–
Gentiobiose	+	v	+	+	v	ND	v	v	ND	v	+	–
Sorbitol	+	–	–	–	–	ND	+	–	ND	v	–	–
Turanose	+	v	+	+	v	ND	+	v	ND	+	+	+
Galactose	+	+	+	+	v	ND	v	–	ND	+	v	–
Mannose	–	+	v	–	v	ND	+	v	ND	+	+	+
Rhamnose	+	+	+	v	–	ND	+	–	ND	v	v	–
D-Tagatose	–	v	v	v	–	ND	+	–	ND	v	–	–
Raffinose	–	v	+	–	–	ND	+	–	ND	+	+	–
Lactose	+	+	+	+	–	ND	v	–	ND	v	+	–
Melezitose	–	+	v	–	–	ND	+	–	ND	v	+	–
N-Acetylglucosamine	+	+	+	–	+	ND	+	+	ND	+	–	+
D-Mannitol	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycerol	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Arabitol	+	v	v	–	v	ND	v	–	ND	v	–	–
Xylitol	+	v	–	–	v	ND	+	–	ND	–	–	–
Hydrolysis of:												
Casein	+	+	+	+	+	v	+	+	+	–	+	+
Hippurate	–	v	–	–	–	+	–	+	+	–	–	+
Tween 20	–	v	v	–	–	v	–	–	–	–	–	–
Tween 40	–	+	+	v	+	v	–	v	+	+	v	–
Tween 60	–	+	+	v	+	+	–	v	+	+	v	–

* The result is strain dependent, and only strain GTC 854^T is positive.

Fig. 1. Morphological characteristics of *B. okuhidensis* GTC 854^T. (top) Phase-contrast micrograph showing endospores. (bottom) Transmission electron micrograph showing flagella. Bars, 5 µm.

carried out with a transmission electron microscope (JEM-100SX; JEOL) (Yamagata, 1993). In the exponential growth phase, the cells of both isolates were Gram-positive rods 0.5–1.0 µm in diameter and 5–7 µm in length; however, they stained Gram-negative in the stationary phase of growth. These organisms showed linked structures and produced oval endospores located at terminal positions (Fig. 1, top). Electron microscopic observation showed that the cells of strain GTC 854^T had flagella only at the ends of the rods (Fig. 1, bottom), whereas cells of strain GTC 855 had many long flagella over the whole surface (data not shown).

Hydrolysis of gelatin, casein, starch, hippurate and Tween 20, 40 and 60, oxidase and catalase reactions, reduction of nitrate, and deamination of phenylalanine were determined as described by Barrow & Feltham (1993) and Yumoto *et al.* (1998). Utilization of carbohydrates was determined with the Biolog automatic identification system, according to the manufacturer's recommendations (Biolog). Most of the phenotypic properties are shown in Table 1 and are compared with those of related alkaliphilic *Bacillus* species. Catalase and oxidase reactions were positive in both of the strains isolated.

Cellular fatty acid composition was analysed as described by Komagata & Suzuki (1987). We analysed strains GTC 854^T, GTC 855 and 10 type strains of related *Bacillus* species (including *Bacillus halodurans* DSM 497^T, *B. alcalophilus* JCM 5262^T, *Bacillus pseudoalcaliphilus* DSM 8725^T, *Bacillus pseudofirmus* DSM 8715^T, *Bacillus clausii* DSM 8716^T, *Bacillus gibsonii* DSM 8722^T, *Bacillus clarkii* DSM 8720^T, *Bacillus halmopalus* DSM 8723^T, *Bacillus horikoshii* DSM 8719^T and *Bacillus agaradhaerens* DSM 8721^T). The cellular fatty acids of strains GTC 854^T and GTC 855 consisted of C_{14:0} (0.6 and 1.2%), C_{16:0} (3.7 and 4.3%), iso-C_{14:0} (1.6 and 1.9%), iso-C_{15:0} (43.2 and 44.2%), iso-C_{16:0} (3.7 and 3.0%), iso-C_{17:0} (7.4 and 5.2%), anteiso-C_{15:0} (25.3 and 26.2%), anteiso-C_{17:0} (9.3 and 5.9%), respectively. The branched saturated fatty acids iso-C_{15:0} and anteiso-C_{15:0} represented the major cellular fatty acids in all strains tested. It was difficult to distinguish the novel isolates from *B. alcalophilus* or *B. pseudoalcaliphilus* by analysis of fatty acid composition. However, the two isolated strains could be distinguished from *B. halodurans* on the basis of significant differences in the iso-C_{15:0} fatty acid content (*B. halodurans*, 29.4%).

To determine the phylogenetic positions of the novel bacteria, the 16S rRNA genes (corresponding to positions 8–1485 in the *Escherichia coli* gene) were PCR-amplified and sequenced as described previously (Ezaki *et al.*, 1994). A comparison with the sequences of all established *Bacillus* species and related low-G + C-content genera indicated that both strains clustered in the genus *Bacillus*, and 16S rDNA sequence similarities with the genus *Bacillus* were above 90% (data not shown). The highest 16S rDNA sequence similarity was with *B. halodurans* (99.3%). They also exhibited higher levels of similarity with *B. alcalophilus* and *B. pseudoalcaliphilus* (96.4 and 95.2%). The sequence similarity between strains GTC 854^T and GTC 855 was 99.7%. A phylogenetic tree based on an almost complete 16S rDNA sequence revealed the relationships between the isolated strains and established *Bacillus* species, and supported the view that the isolated strains are members of the genus *Bacillus* (Fig. 2). The tree was constructed using the CLUSTAL W software (Thompson *et al.*, 1994), and phylogenetic distances were calculated by using the neighbour-joining method (Saitou & Nei, 1987).

DNA was extracted and purified as described by Ezaki *et al.* (1994). DNA base composition was determined with the HPLC method (Ezaki *et al.*, 1990). Purified DNA (5–10 µg) dissolved in 10 µl distilled water was denatured by boiling for 10 min. Ten microlitres nuclease P1 (2 U ml⁻¹) was added and the mixture was incubated at 50 °C for 1 h. Ten microlitres of alkaline phosphatase (2.4 U ml⁻¹) was then added and the mixture was incubated at 37 °C for 30 min. Aliquots (5–10 µl) of the sample were analysed by HPLC. The G + C contents of the DNAs of strains GTC 854^T and GTC 855 were 41.0 and 41.1 mol %, respectively; those of the reference strains *B. halodurans* DSM 497^T, *B.*

Fig. 2. Phylogenetic positions of *B. okuhidensis* strains GTC 854^T and GTC 855 within the species *Bacillus*. The 16S rDNA sequences used in the tree were obtained from the DDBJ database. *Staphylococcus aureus* was used as the outgroup strain. The values indicate the percentages of occurrence in 1000 bootstrapped trees. The scale bar represents one base change per 100 bases.

alcalophilus JCM 5262^T and *B. pseudocalcalophilus* DSM 8725^T were 42.8, 38.5 and 39.1 mol%, respectively.

According to 16S rRNA gene sequence analysis, the two isolated strains were closely related to *B. halodurans*, *B. alcalophilus* and *B. pseudocalcalophilus*. Chromosomal DNA similarity values among strain GTC 854^T, strain GTC 855, *B. halodurans* DSM 497^T, *B. alcalophilus* JCM 5262^T and *B. pseudocalcalophilus* DSM 8725^T were estimated. Reciprocal DNA–DNA hybridization was performed as described by Ezaki *et al.* (1989). Hybridization was carried out under stringent conditions (2 × SSC, 50% deionized formamide hybridization solution) at 41 °C for 6 h. The plates were read with a Titretek Fluoroskan II spectrophotometer (excitation at 360 nm, emission at 450 nm). DNA relatedness results indicated that the two isolates were tightly related (showing more than 96% similarity). Both strain GTC 854^T and strain GTC 855 showed less than 22% DNA similarity to *B. alcalophilus* JCM 5262^T and *B. pseudocalcalophilus* DSM 8725^T. DNA similarities between the isolated strains and *B. halodurans* DSM 497^T ranged from 36.9 to 45.2%.

In accord with the phylogenetic relationship based on 16S rDNA sequence similarity, the two isolates were placed in a single species of genus *Bacillus*. Most of these 16S rDNA sequence similarities were less than 97%; the only exception was the level of similarity with *B. halodurans* (99.3%). Stackebrandt & Goebel (1994) stated that levels of similarity between 16S rDNA sequences that are less than 97% suggest that the strains do not belong to the same species. However, DNA–DNA hybridization distinguished the isolated strains from *B. halodurans* (DNA similarity values of 36.9–45.2%) and *B. alcalophilus* and *B. pseudocalcalophilus* (DNA relatedness of less than 22%). The DNA–DNA hybridization results also support the view that the novel isolates belong to the same DNA-similarity group, because the similarity value between the two isolated strains was greater than 96%. The isolates can also be distinguished from established *Bacillus* species on the basis of a combination of the physiological and phenotypic properties shown in Table 1. Currently, DNA reassociation analysis represents the best applicable procedure used in species definition. Wayne *et al.* (1987) concluded that the

phylogenetic definition of a species would generally include strains with at least 70% DNA–DNA relatedness, and phenotypic characteristics should agree with this definition. All of these results confirmed that the isolated strains should be classified in a new species in the genus *Bacillus*, for which we propose the name *Bacillus okuhidensis*, the type strain being GTC 854^T (= JCM 10945^T = DSM 13666^T).

Description of *Bacillus okuhidensis* sp. nov.

Bacillus okuhidensis (ok.u.hid.en'sis. N.L. masc. adj. *okuhidensis* pertaining to Okuhida in Gifu, Japan, where the strains were initially isolated).

Cells are rod-shaped with a length of approx. 5–7 µm and a width of 0.5–1.0 µm. Cells are motile by means of peritrichous flagella, and produce terminally located ellipsoidal spores. Colonies are circular, convex, smooth and yellowish. Cells stain slightly Gram-positive in the exponential growth phase but Gram-negative in the stationary growth phase. Catalase and oxidase reactions are positive. The temperature range for growth is 30–60 °C, the optimum being 45–50 °C. The pH range for growth is 6.0–11.0, the optimum being pH 10.5. Grows at 10% NaCl (w/v). Branched saturated fatty acids iso-C_{15:0} (43.7% ± 0.7%) and anteiso-C_{15:0} (25.8% ± 0.6%) represent the major cellular fatty acids. Nitrate is reduced to nitrite. Phenylalanine is not deaminated. Casein, starch and gelatin are hydrolysed. Hippurate and Tween 20, 40 and 60 are not hydrolysed. Utilizes salicin, glycogen, inositol, melibiose, gentiobiose, L-arabinose, sorbitol, D-xylose, turanose, galactose, rhamnose, lactose, D-mannitol, glycerol, arabinol, N-acetylglucosamine and xylitol, but not mannose, D-tagatose, raffinose or melezitose (utilization data were obtained with Biolog). The G + C content of the DNA is 41.0–41.1 mol% (determined by HPLC). The type strain is GTC 854^T (= JCM 10945^T = DSM 13666^T).

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