Bacillus polygoni sp. nov., a moderately halophilic, non-motile obligate alkaliphile isolated from indigo balls

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A moderately halophilic, obligate alkaliphile (growth range pH 8–12), designated strain YN-1^T, was isolated from indigo balls obtained from Ibaraki, Japan. The cells of the isolate stained Gram-positive, and were aerobic, non-motile, sporulating rods which grew optimally at pH 9. The strain grew in 3–14 % NaCl with optimum growth in 5 % NaCl. It hydrolysed casein and Tweens 20, 40 and 60, but not gelatin, starch, DNA or pullulan. Its major isoprenoid quinone was MK-7 and its cellular fatty acid profile mainly consisted of anteiso-C_{15:0}, anteiso-C_{17:0} and anteiso-C_{17:1}. 16S rRNA phylogeny suggested that strain YN-1^T was a member of group 7 (alkaliphiles) of the genus *Bacillus*, with the closest relative being *Bacillus clarkii* DSM 8720^T (similarity 99.5 %). However, DNA–DNA hybridization showed a low DNA–DNA relatedness (7 %) of strain YN-1^T with *B. clarkii* DSM 8720^T. Owing to the significant differences in phenotypic and chemotaxonomic characteristics, and phylogenetic and DNA–DNA relatedness data, the isolate merits classification as a new species, for which the name *Bacillus polygoni* is proposed. The type strain of this species is YN-1^T (=JCM 14604^T=NCIMB 14282^T).

Alkaliphilic *Bacillus* species have been studied in terms of their physiological adaptation to high pH, including their bioenergetic properties (Goto *et al.*, 2005; Krulwich *et al.*, 2007), ion transport systems (Krulwich *et al.*, 2007) and cell wall components (Aono *et al.*, 1999). Most of these studies were performed using a limited number of strains representative of alkaliphiles. However, studies of the cytochrome content (Yumoto *et al.*, 1997), lipid composition (Clejan *et al.*, 1986) and cell wall composition (Aono & Horikoshi, 1983) of various alkaliphilic *Bacillus* strains have indicated that the environmental adaptation mechanisms of alkaliphilic *Bacillus* strains differ. Therefore, it is

considered that such *Bacillus* strains have diverse physiological alkaline adaptation mechanisms because they exhibit taxonomic diversity and have a wide ecological distribution.

Nearly 30 alkaliphilic *Bacillus* species have been identified to date (Nielsen *et al.*, 1995; Nogi *et al.*, 2005; Yumoto, 2007). These species are not scattered phylogenetically, but are grouped into three main clusters on the basis of their 16S rRNA gene sequence. Alkaliphiles exist not only in specific environments such as alkaline soda lakes (Duckworth *et al.*, 1996), but also in ordinary soils and faeces (Horikoshi, 1991). Some alkaliphiles can thrive in different alkaline environments, whereas some alkaliphiles can thrive only in specific environments. Furthermore, some alkaliphiles can thrive in diverse environments regardless of ambient pH. Such species may differ in their physiological functions for adapting to the ambient environment, even among strains of the same species.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence reported in this paper is AB292819.

Supplementary figures showing maximum-parsimony and minimum-evolution phylogenetic trees are available with the online version of this paper.

For example, although alkaliphilic *Bacillus* species isolated from the gut of higher termites exhibit similar taxonomic characteristics to a *Bacillus* strain isolated from soil, some of them show distinct sensitivity to NaCl compared to the corresponding soil isolate (Thongaram *et al.*, 2003).

Since the original isolation of strain YN-1^T from an indigo ball, the NADH dehydrogenase (Xu *et al.*, 1991; Kitazume *et al.*, 2006), cyanide-insensitive non-proteinaceous respiratory material (Higashibata *et al.*, 1998), flagella rotatory system (Hirota & Imae, 1983) and polyamines (Hamasaki *et al.*, 1993) of this strain have been studied. On the basis of these data, it is important to study the phylogenetic position of this alkaliphilic isolate. In consideration of the relationship between the taxonomic characteristics and physiological function of alkaliphiles, their characterization and identification are crucial. In the present study phenotypic and chemotaxonomic characteristics have been used together with phylogenetic analysis based on the 16S rRNA gene sequence of strain YN-1^T to show that the new isolate merits classification as a novel species of *Bacillus*.

Strain YN-1^T was originally isolated and identified as a *Bacillus* species by Ota *et al.* (1975). In the present study, PYA (peptone/yeast extract/alkaline) agar plates (pH 10) containing 8 g peptone (Kyokuto), 3 g yeast extract (Merck) and 15 g agar in 1 l NaHCO₃/Na₂CO₃ buffer (100 mM in deionized water; pH 10) (Yumoto *et al.*, 1998) was used as the basal medium for the strain. Cells for chemotaxonomic analyses were cultured with reciprocal shaking (120 r.p.m.) at 27 °C for 18 h. In addition to strain YN-1^T, *Bacillus clarkii* DSM 8720^T, *Bacillus agaradhaerens* DSM 8721^T and *Bacillus cellulosilyticus* JCM 9156^T were used as reference strains for DNA–DNA hybridization. These were cultured using PYA broth containing 100 mM NaHCO₃/Na₂CO₃ buffer (pH 10).

For phenotypic characterization, PYA medium was also used as the basal medium. The culture was incubated at 27 °C for 2 weeks and the experiment was performed three times. Acid production from carbohydrate was determined by the method of Hugh & Leifson (1953) using thymol blue (0.008 %, w/v) instead of bromthymol blue at pH 10. Growth experiments at pH 7–10 were performed using PYA medium containing 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7–8), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10), Na₂HPO₄/NaOH buffer (pH 11) or KCl/NaOH buffer (pH 12). Hydrolysis of cellulose and xylan was examined according to the method of Teather & Wood (1982) using medium containing 1 % substrate in the basal medium. Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998) and Barrow & Feltham (1993).

To observe negatively stained cells by TEM, strain YN-1^T cells were grown on a PYA agar slant. The procedures for TEM preparation and observation were as described previously (Yumoto *et al.*, 2001). The morphological, physiological and biochemical characteristics of the isolate are given in the species description. The isolate

was Gram-positive and produced ellipsoidal spores centrally positioned within a sporangium which was not swollen. TEM showed that the cells were non-flagellate rods (0.4–0.5 × 1.0–3.5 µm).

Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto *et al.*, 1998, 2001). The results of GLC of the fatty acids of strain YN-1^T grown at pH 10 are shown in Table 1. The fatty acids were mostly of the branched type. Differences were observed between YN-1^T and its closest neighbour, *B. clarkii* DSM 8720^T. The major isoprenoid quinone was MK-7.

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA base composition was determined by the method of Tamaoka & Komagata (1984). The DNA G + C content of strain YN-1^T was 42.9 mol%, which is similar to that of *B. clarkii* DSM 8720^T (41.6 mol%)

The 16S rRNA gene was amplified by PCR using primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'). The approximately 1.5 kb PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 3100; Applied Biosystems). Multiple alignments of the sequences were performed using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining (Kimura, 1980; Saitou & Nei, 1987), maximum-parsimony and minimum-evolution methods in MEGA4 (Tamura *et al.*, 2007). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. The similarity between

Table 1. Fatty acid composition (%) of *B. polygoni* YN-1^T and *B. clarkii* DSM 8720^T grown at pH 10

ND, Not detected. Both sets of data are based on the results of this study.

Fatty acid	<i>B. polygoni</i>	<i>B. clarkii</i>
iso-C _{14:0}	1.9	2.2
C _{14:0}	0.4	1.0
iso-C _{15:0}	10.7	69.1
anteiso-C _{15:0}	43.7	20.6
iso-C _{16:0}	4.1	1.0
C _{16:0}	0.7	1.1
iso-C _{17:0}	2.7	2.7
anteiso-C _{17:0}	10.5	1.5
C _{16:1}	3.2	ND
iso-C _{17:1}	2.6	ND
anteiso-C _{17:1}	16.3	ND
Other	3.3	0.9
Total unsaturated	22.1	ND
Total branched	92.5	97.1
Total anteiso-branched	70.5	22.1
Total iso-branched	22.0	75.0
anteiso-/iso-	3.20	0.29

sequences was calculated using the GENETYX computer program (Software Development). The 16S rRNA gene sequence (1558 bp) of strain YN-1^T was obtained. Similarity with previously reported strains was determined, and a phylogenetic tree was constructed (Fig. 1 and supplementary Figs S1 and S2, available with the online version of this paper). These results suggest that strain YN-1^T is a member of group 7 (alkaliphiles) of the genus *Bacillus* (Nielsen *et al.*, 1994). The strain showed the highest similarity with *B. clarkii* DSM 8720^T (99.5 %) among the strains identified as species. It also showed high sequence similarities with other phylogenetic neighbours, namely *B. agaradhaerens* DSM 8721^T (96.1 %) and *B. cellulosilyticus* JCM 9156^T (95.6 %).

The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and black microplates. The hybridization temperature was 38.1 °C. According to the sequence similarities and phylogenetic analysis based on the 16S rRNA gene sequence, strain YN-1^T is most closely related to *B. clarkii* DSM 8720^T among the strains identified as species. Therefore, DNA–DNA hybridizations between strain YN-1^T and *B. clarkii* DSM 8720^T, *B. agaradhaerens* DSM 8721^T and *B. cellulosilyticus* JCM 9156^T were performed. DNA–DNA hybridization data indicated that the isolate is distinct from *B. clarkii* DSM 8720^T (7 %), *B. agaradhaerens* DSM 8721^T (8 %) and *B. cellulosilyticus* JCM 9156^T (10 %). Therefore, it is proposed that strain YN-1^T represents a novel species.

Strain YN-1^T can also be differentiated from *B. clarkii* DSM 8720^T and other phylogenetic neighbours on the basis of several phenotypic and chemotaxonomic characteristics (Table 2).

Table 2. Characteristics differentiating *B. polygoni* YN-1^T from other alkaliphilic *Bacillus* species

1, *Bacillus polygoni*; 2, *Bacillus clarkii*; 3, *Bacillus agaradhaerens*; 4, *Bacillus cellulosilyticus*; 5, *Bacillus oshimensis*. +, Positive; –, negative; ±, slightly swollen to not swollen; ND, no data. The characteristics of other alkaliphilic *Bacillus* species are from Nielsen *et al.* (1995), Yumoto *et al.* (2005), Nogi *et al.* (2005) and this study.

Characteristic	1	2	3	4	5
Swollen sporangia	–	+	+	+	±
Oxidase	–	+	ND	–	+
Hydrolysis of:					
Casein	+	+	–	–	+
Gelatin	–	+	–	–	+
Starch	–	–	–	+	+
Nitrate reduction	–	+	+	+	–
Growth at:					
45 °C	+	+	+	–	–
pH 12	+	–	ND	–	ND
0 % NaCl	–	+	+	+	+

There are several contradictions between the present study and previous reports on strain YN-1^T. First, although Higashibata *et al.* (1998) reported that strain YN-1^T possesses *caa*₃-type cytochrome *c* oxidase, the oxidase reaction was negative in our study. This may be due to a greater dependence on cyanide-insensitive non-proteinaceous material than on cytochrome *c* oxidase for oxygen metabolism in this strain. Although most alkaliphilic *Bacillus* strains are aerobes, Nogi *et al.* (2005) reported that several alkaliphilic *Bacillus* species are oxidase-negative. This suggests that several alkaliphilic *Bacillus*

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence data of *B. polygoni* strain YN-1^T and other related organisms. The grouping is according to Nielsen *et al.* (1994). Bootstrap percentages (based on 1000 replicates) greater than 50 % are shown at branch points. Bar, 0.01 changes per nucleotide position.

species have an alternative system, such as one based on cyanide-insensitive non-proteinaceous material. Second, although Hirota & Imae (1983) reported on Na⁺-driven flagellar motors in strain YN-1^T, we found that the strain is non-motile and has no flagella. This may be explained by considering that extensive reisolation had been performed before the study by Higashibata *et al.* (1998), and we have studied the reisolated strain.

Strain YN-1^T exhibited notable growth at pH 12. To the best of our knowledge, this is the first report of an alkaliphilic *Bacillus* species that can grow at pH 12. This is understandable because the strain was isolated from indigo fermentation fluid and the initial stage of indigo fermentation is carried out at a high pH (approx. pH 12).

On the basis of the above results, it is suggested that the isolate is a novel species for which the name *Bacillus polygoni* sp. nov. is proposed.

Description of *Bacillus polygoni* sp. nov.

Bacillus polygoni (po.ly'gon.i. N.L. gen. n. *polygoni* of *Polygonum tinctorium*, referring to polygonum, the fermentation product from which the type strain was isolated).

The strain is a non-motile, Gram-positive, aerobic and straight rod (0.4–0.5 × 1.0–3.5 µm) and produces centrally located ellipsoidal spores that do not swell the sporangium. Colonies are circular and white. The isolate grows at 3–14 % NaCl. The growth temperature range is 5–47 °C with an optimum growth temperature range of 29–31 °C at pH 10. The maximum generation time was 45 min at 35–39 °C. pH range for growth is pH 8–12 with an optimum of pH 9. Positive for catalase, ONPG and nitrate reduction; negative for oxidase, indole production, deamination of phenylalanine and H₂S production. Positive for hydrolysis of casein and Tweens 20, 40 and 60. Negative for hydrolysis of gelatin, starch, xylan, cellulose, MUG (4-methylumbelliferone glucuronide), aesculin, DNA, Tween 80, pullulan and hippurate. Acid, but no gas, is produced from D-xylose, D-arabinose, D-glucose, D-fructose, maltose, D-mannose, raffinose, glycerol, D-mannitol, sucrose and myo-inositol when grown at pH 10. No acid is produced from D-galactose, lactose, melibiose, sorbitol, rhamnose, salicin, xylitol and meso-erythritol. The major isoprenoid quinone is MK-7. The major cellular fatty acids consist of anteiso-C_{15:0}, anteiso-C_{17:0} and anteiso-C_{17:1}. The DNA G + C content is 42.9 mol%.

The type strain, YN-1^T (=JCM 14604^T=NCIMB 14282^T), was originally isolated from indigo balls from Ibaraki, Japan.

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