

## *Bacillus krulwichiae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and *m*-hydroxybenzoate

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Obligate alkaliphilic strains, AM31D<sup>T</sup> and AM11D, that utilize benzoate and *m*-hydroxybenzoate were isolated from soil obtained from Tsukuba, Ibaraki, Japan. The isolates grew at pH 8–10, but not at neutral pH. They were Gram-positive, facultatively anaerobic, straight rods with peritrichous flagella and produced ellipsoidal spores. The isolates reduced nitrate to nitrite and grew in 0–14% NaCl, but not in higher concentrations. The major isoprenoid quinones were menaquinone-5, -6 and -7, and the cellular fatty acid profile consisted of significant amounts of 15-C branched-chain acids, isoC<sub>15:0</sub> and anteisoC<sub>15:0</sub>. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that strain AM31D<sup>T</sup> was a member of group 6 (alkaliphiles) in the genus *Bacillus*. DNA–DNA hybridization revealed a low relatedness of the isolates with several phylogenetically close neighbours, including *Bacillus alcalophilus* and *Bacillus pseudocaliphilus* (less than 19.3%). Based on phenotypic characteristics, phylogenetic data and DNA–DNA relatedness data, it was concluded that these isolates merited classification as a new species, for which the name *Bacillus krulwichiae* is proposed. The type strain of this species is AM31D<sup>T</sup> (=NCIMB 13904<sup>T</sup>=JCM 11691<sup>T</sup>=IAM 15000<sup>T</sup>).

Alkaliphilic *Bacillus* spp. have been isolated to investigate their physiological adaptation to high pH and to utilize their enzymes industrially. Although considered to be extremophiles, alkaliphilic *Bacillus* spp. are distributed not only in unique places such as alkaline soda lakes (Duckworth *et al.*, 1996), but also in ordinary soils and faeces (Horikoshi, 1991). Alkaliphilic bacteria can be either facultative or obligate alkaliphiles (Krulwich & Guffanti, 1989). Numerous alkaliphilic micro-organisms have been isolated and more than 16 species of alkaliphilic *Bacillus* spp., including facultative and obligate types, have been identified to date

(Vedder, 1934; Spanka & Fritze, 1993; Nielsen *et al.*, 1995; Agnew *et al.*, 1995; Fritze, 1996; Switzer Blum *et al.*, 1998; Yumoto *et al.*, 1998).

The microbial degradation of aromatic compounds is important not only for the industrial applications of the respective enzymes involved, but also for studying structural, functional and genetic aspects of aromatic compound oxygenases. However, there is little information concerning alkaliphiles that degrade aromatic compounds, or concerning aromatic compound oxygenases isolated from them. Isolation of aromatic-compound-degrading alkaliphiles might enable the acquisition of new information not only on the taxonomy, physiology and enzymology of alkaliphiles, but also on aromatic compound oxygenases (Gibson & Subramanian, 1984; Mason & Cammack, 1992).

In the present study, obligately alkaliphilic micro-organisms that can degrade benzoate and *m*-hydroxybenzoate were

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Abbreviations: AMBS, alkaline mineral basal salt; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank accession number for the 16S rRNA sequence of strain AM31D<sup>T</sup> is AB086897.

isolated to study the degradation of aromatic compounds in alkaline environments and the aromatic compound oxygenases that are active at high pH values. Phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequences showed that the new isolates merited classification as a novel *Bacillus* species.

Aromatic-compound-contaminated garden soil samples obtained from Tsukuba (36° 7' N, 140° 13' E), Ibaraki, Japan, were added to 10 ml alkaline mineral basal salt (AMBS) medium (pH 10) containing 0.2 g yeast extract, 3 g hydroxybenzoate, 2.5 g NH<sub>4</sub>NO<sub>3</sub>, 1.5 g K<sub>2</sub>HPO<sub>4</sub>, 1.5 g Na<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mg MnSO<sub>4</sub>·nH<sub>2</sub>O, 0.5 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O and 10 g Na<sub>2</sub>CO<sub>3</sub> per litre in large tubes (25 × 200 mm) and incubated aerobically at 30 °C for 48 h. Enrichments (0.5 ml) were transferred to fresh medium and cultured for 24 h, then plated onto AMBS agar plates. The isolates were reisolated five times and maintained in PYA (peptone/yeast extract/alkaline) agar medium consisting of 8 g peptone (Kyokuto), 3 g yeast extract (Merck), 15 g agar, 1 g K<sub>2</sub>HPO<sub>4</sub>, 3.5 mg EDTA, 3 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg MnSO<sub>4</sub>·nH<sub>2</sub>O, 1 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 2 mg Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O and 1 mg H<sub>3</sub>BO<sub>3</sub> in 1 l NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (100 mM in deionized water; pH 10) at 27 °C. Cells for chemotaxonomic analysis were harvested in the late exponential phase after cultivation with reciprocal shaking (140 r.p.m.) at 27 °C in PYA medium. In addition to these isolates, *Bacillus alcalophilus* JCM 5262<sup>T</sup> and *Bacillus pseudocaliphilus* DSM 8725<sup>T</sup> were used as reference strains for DNA–DNA hybridization. These micro-organisms were cultivated using PYA broth at 27 °C.

For the phenotypic characterization, PYA medium was used as the basal medium. The culture was incubated at 27 °C for 2 weeks and the experiment was performed more than twice. Acid production from carbohydrate was determined by the method of Hugh & Leifson (1953) using thymol blue instead of bromothymol blue at pH 10. Growth experiments at pH 7–10 were performed using PYA media containing 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7–8) or 100 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9–10). Anaerobic growth was tested in PYA broth (pH 10) by substituting air with N<sub>2</sub> gas. Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998) and as described in Barrow & Feltham (1993).

For observation of negatively stained cells by transmission electron microscopy (TEM) and for platinum- and palladium-coated cells by scanning electron microscopy (SEM), cells were grown on a PYA agar slant. The procedure for TEM and SEM preparations and observations were performed as described previously (Yumoto *et al.*, 2002).

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2001).

Bacterial DNA was prepared according to the method of Marmur (1961). DNA base composition was determined by

the method of Tamaoka & Komagata (1984). 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.

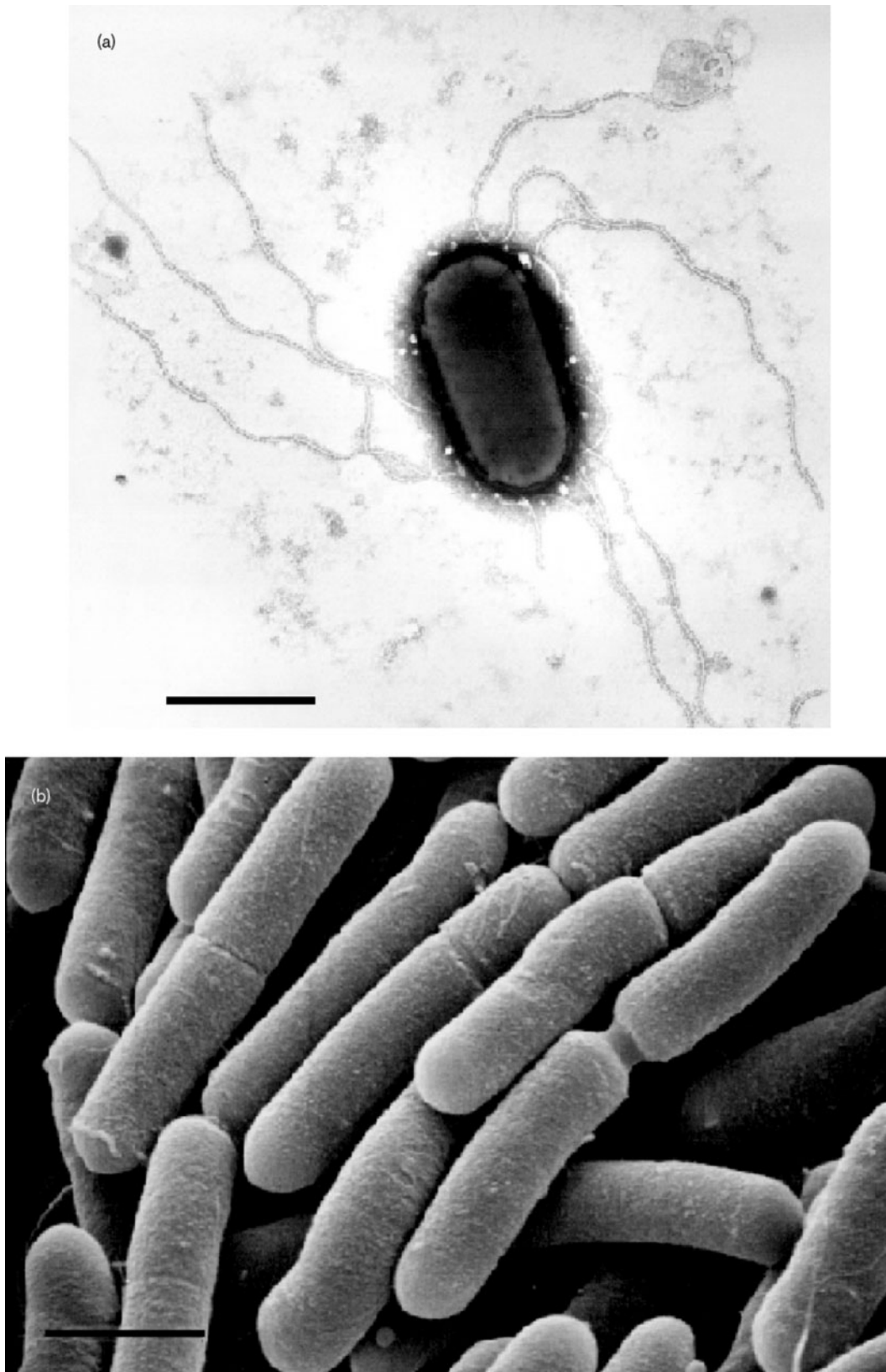
The 16S rRNA gene sequence corresponding to position 27–1519 in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978) was amplified by PCR. The approximately 1.5 kb PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 377; Applied Biosystems). Multiple alignments of the sequence were performed and the nucleotide substitution rate ( $K_{nuc}$ ) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson *et al.*, 1994). Similarity values for sequences were calculated using the GENETYX program (Software Development).

Two strains were isolated on AMBS medium (pH 10). These isolates grew better in AMBS broth containing either 0.3 % *m*-hydroxybenzoate or benzoate than in the same medium devoid of the aromatic compounds. Strain AM31<sup>T</sup> grew strongly with both *m*-hydroxybenzoate (OD<sub>650</sub> = 0.87) and benzoate (OD<sub>650</sub> = 0.86) as the substrate. In contrast, AM11D grew better with benzoate (OD<sub>650</sub> = 0.67) than with *m*-hydroxybenzoate (OD<sub>650</sub> = 0.32). These results demonstrated that the two isolates utilized aromatic compounds such as benzoate and *m*-hydroxybenzoate as the substrate. To the best of our knowledge, this is the first observation of alkaliphilic *Bacillus* strains utilizing aromatic compounds.

Colonies of strain AM31D<sup>T</sup> and AM11D on PYA agar were circular and colourless; cells were Gram-positive, peritrichously flagellated rods measuring 0.5–0.7 × 1.5–2.6 μm (Fig. 1) and produced subterminally located ellipsoidal spores. Spores did not cause swelling of the sporangia.

Physiological and biochemical characteristics of the two isolates are given in the species description below. Growth occurred at a similar level at pH values from 8 to 10, but not at pH 7. The two isolates showed quite similar characteristics. They were facultative anaerobic bacteria, differing only in protease activity. The morphological and phenotypic characteristics suggested that the isolates were obligately alkaliphilic members of the genus *Bacillus*.

The isoprenoid quinones extracted from isolates using TLC were analysed by HPLC. Analysis revealed that menaquinone-5, -6 and -7 are the major quinones of the isolates. Similar menaquinone contents occur in *Bacillus halodurans* (Aono, 1995; Takami & Horikoshi, 1999). The ratio of menaquinone-5/-6/-7 was 1:11:27. GLC analysis revealed that the fatty acids of these strains consisted of isoC<sub>15:0</sub>, anteisoC<sub>15:0</sub>, C<sub>16:1</sub>, anteisoC<sub>17:0</sub> and anteisoC<sub>17:1</sub>. Among these, anteisoC<sub>15:0</sub> was the major component, comprising 45.6–49.0% of the total fatty acid content. Results of the analysis of the two isolates are shown in Table 1. Clejan *et al.*



**Fig. 1.** (a) TEM of a negatively stained cell of *Bacillus krulwichiae* AM31D<sup>T</sup>, showing peritrichous flagellation. (b) SEM of platinum/palladium-coated *Bacillus krulwichiae* AM31D<sup>T</sup> cells, showing slightly rough cell surface aspect. Bars, 1 μm.

**Table 1.** Fatty acid composition of *Bacillus krulwichiae* AM31D<sup>T</sup> and AM11D, other alkaliphiles and *Bacillus subtilis*

1, *Bacillus krulwichiae* AM31D<sup>T</sup>; 2, *Bacillus krulwichiae* AM11D; 3, *Bacillus cohnii* DSM 6307<sup>T</sup>; 4, *Bacillus alcalophilus* JCM 5262<sup>T</sup>; 5, *Bacillus subtilis* IAM 1026. Data for 3, 4 and 5 are from Yumoto *et al.* (2000). ND, Not detected. All these alkaliphiles were grown at pH 10 and *Bacillus subtilis* was grown at neutral pH.

| Fatty acids              | 1    | 2    | 3    | 4    | 5    |
|--------------------------|------|------|------|------|------|
| isoC <sub>14:0</sub>     | 2.2  | 1.4  | 2.0  | 0.6  | 1.8  |
| C <sub>14:0</sub>        | ND   | ND   | 0.4  | 0.5  | 0.3  |
| isoC <sub>15:0</sub>     | 17.1 | 19.2 | 25.9 | 31.8 | 29.1 |
| anteisoC <sub>15:0</sub> | 49.0 | 45.6 | 34.6 | 42.9 | 37.9 |
| C <sub>15:0</sub>        | 1.1  | ND   | 0.2  | 0.3  | ND   |
| isoC <sub>16:0</sub>     | 0.8  | 1.7  | 5.3  | 1.0  | 4.9  |
| isoC <sub>16:1</sub>     | 2.1  | 1.3  | 1.3  | 0.2  | 0.1  |
| C <sub>16:0</sub>        | 1.6  | 1.6  | 0.9  | 2.1  | 3.2  |
| C <sub>16:1</sub>        | 9.8  | 13.6 | 3.1  | 1.5  | 0.2  |
| isoC <sub>17:0</sub>     | 2.9  | 3.3  | 4.4  | 4.3  | 11.2 |
| anteisoC <sub>17:0</sub> | 4.9  | 4.5  | 10.0 | 11.2 | 9.6  |
| isoC <sub>17:1</sub>     | 0.7  | 1.1  | 6.9  | 1.7  | 0.3  |
| C <sub>17:0</sub>        | 0.2  | ND   | 0.2  | ND   | ND   |
| anteisoC <sub>17:1</sub> | 4.1  | 4.2  | 4.1  | 0.9  | 0.2  |
| C <sub>18:0</sub>        | ND   | ND   | ND   | ND   | 0.2  |
| C <sub>18:1</sub>        | ND   | ND   | 0.1  | ND   | ND   |
| Others                   | 3.7  | 2.6  | 0.7  | 0.4  | 1.1  |
| Total unsaturated        | 16.7 | 20.2 | 15.5 | 4.3  | 0.8  |
| Iso-branched             | 25.8 | 28.0 | 45.8 | 39.6 | 47.4 |
| Anteiso-branched         | 58.0 | 54.3 | 48.7 | 55.0 | 47.7 |
| Ratio anteiso: iso       | 2.25 | 1.94 | 1.06 | 1.39 | 1.01 |
| Total branched           | 83.8 | 82.3 | 94.5 | 94.6 | 95.1 |

(1986) reported that obligate alkaliphilic *Bacillus* spp. contain a high concentration of unsaturated fatty acids. The isolates also contained a larger amount of unsaturated fatty acids than *Bacillus subtilis*, the facultatively alkaliphilic *Bacillus cohnii* and the obligately alkaliphilic *Bacillus alcalophilus*. The menaquinone and fatty acid contents of the new isolates are characteristic of the genus *Bacillus*.

The 16S rRNA gene sequence of strain AM31D<sup>T</sup> was analysed to determine its phylogenetic position. The sequence of 1507 bases of the 16S rRNA gene of strain AM31D<sup>T</sup> was compared with those of 11 other alkaliphilic *Bacillus* and two neutrophilic or alkaliphilic species belonging to other related taxa. The phylogenetic tree constructed using the neighbour-joining method (Fig. 2) and 16S rRNA gene sequence similarity (data not shown) showed that strain AM31D<sup>T</sup> was a member of the *Bacillaceae*. Strain AM31D<sup>T</sup> was placed in group 6 (alkaliphiles) (Nielsen *et al.*, 1994). The highest similarity value was observed with the obligate alkaliphiles (Nielsen *et al.*, 1995) *Bacillus alcalophilus* (94.7%) and *Bacillus pseudalcaliphilus* (94.8%). These results and the demonstrated obligate alkaliphilic nature of AM31D<sup>T</sup> were consistent with the phylogenetic placement of the isolates.

The DNA G+C contents of strains AM31D<sup>T</sup> and AM11D were 41.5 and 40.6 mol%, respectively. These values fell within the defined range of the genus *Bacillus* and were similar to those of the phylogenetically related alkaliphilic *Bacillus* species.

According to the results of the 16S rRNA gene sequence analysis, strain AM31D<sup>T</sup> was closely related to *Bacillus alcalophilus* and *Bacillus pseudalcaliphilus*. Therefore, the level of DNA–DNA relatedness between strains AM31D<sup>T</sup> and AM11D, and the closely related strains given above were estimated. DNA–DNA relatedness results indicated that the two isolates were closely related to each other (more than 94.7% similarity) and different (9.5–19.3% similarity) from *Bacillus alcalophilus* and *Bacillus pseudalcaliphilus* (Table 2).

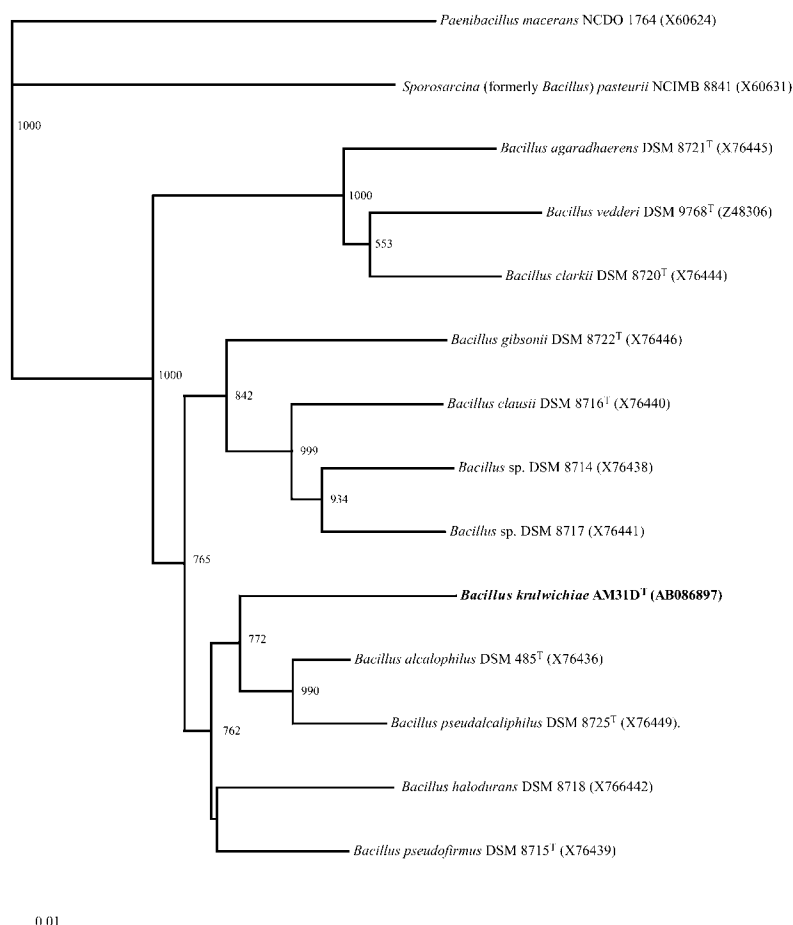
*Bacillus* strains AM31D<sup>T</sup> and AM11D differed from other relatively closely related species in terms of phenotypic characteristics as follows: *Bacillus alcalophilus* grows at 10 °C, does not grow in 10% NaCl and has a DNA G+C content of 36.2–38.4 mol%; *Bacillus pseudalcaliphilus* neither hydrolyses Tween 20 nor reduces nitrate, and grows at 10 °C; *Bacillus pseudofirmus* does not hydrolyse Tween 20, deaminates phenylalanine and grows at 10 °C; and *Bacillus halodurans* grows at 50 °C (Table 3).

On the basis of the above results, the two isolates were designated as a new species for which name *Bacillus krulwichiae* sp. nov. is proposed; the type strain is AM31D<sup>T</sup>.

### Description of *Bacillus krulwichiae* sp. nov.

*Bacillus krulwichiae* (krul.wich.i'ae N.L. fem. gen. n. *krulwichiae* of Krulwich; named after American microbiologist Terry A. Krulwich who made fundamental contributions to the study of alkaliphilic bacteria).

Cells are Gram-positive peritrichously flagellated straight rods (0.5–0.7 × 1.5–2.6 μm) and produce subterminally located ellipsoidal spores. Spores do not cause swelling of sporangia. Both aerobic and anaerobic growth is observed. Colonies are circular and colourless. Catalase and oxidase reactions are positive. Negative for indole production, ONPG hydrolysis and H<sub>2</sub>S production. Growth occurs at pH 8–10 at almost equal intensity, but not at pH 7. Grows at 14% NaCl, but not at higher concentrations. Nitrate is reduced to nitrite. Acid, but no gas, is produced from D-xylose, D-glucose, D-fructose, D-galactose, D-ribose, maltose, sucrose, trehalose, glycerol and mannitol when grown at pH 10. No acid is produced from D-arabinose, L-rhamnose, D-mannose, lactose, cellobiose, melibiose, raffinose, *myo*-inositol and sorbitol. Positive for hydrolysis of starch, DNA, hippurate and Tween 20, 40, 60 and 80. Hydrolysis of casein and gelatin is variable among strains. Utilizes benzoate and *m*-hydroxybenzoate as sole carbon sources. The major isoprenoid quinones are menaquinone-5, -6 and -7. isoC<sub>15:0</sub> (17.1–19.2%) and anteisoC<sub>15:0</sub> (45.6–49.0%) represent the main fatty acids



**Fig. 2.** Phylogenetic tree derived from 16S rRNA gene sequence data of *Bacillus krulwichiae* AM31D<sup>T</sup>, other alkaliphilic *Bacillus* spp. and other related organisms using the neighbour-joining method. Numbers indicate bootstrap values greater than 500. Bar, 0.01  $K_{nuc}$  units.

produced during growth in an alkaline medium (pH 10). The DNA G + C content is 40.6–41.5 mol%, as determined by HPLC. Strains AM31D<sup>T</sup> and AM11D were isolated from a soil sample obtained from Tsukuba, Ibaraki, Japan.

**Table 2.** DNA base composition and levels of relatedness between *Bacillus krulwichiae* AM31D<sup>T</sup> and AM11D and DNAs of other closely related alkaliphilic *Bacillus* strains

| Strain  | G+C content (mol%)* | Reassociation (%) with biotinylated DNA from: |       |
|---|---------------------|---|-------|
|   |                     | AM31D <sup>T</sup>                            | AM11D |
| <i>Bacillus krulwichiae</i> AM31D <sup>T</sup>          | 41.5                | 100   | 96.5  |
| <i>Bacillus krulwichiae</i> AM11D                       | 40.6                | 94.7  | 100   |
| <i>Bacillus alcalophilus</i> JCM 5262 <sup>T</sup>      | 37.3                | 19.3  | 9.5   |
| <i>Bacillus pseudalcaliphilus</i> DSM 8725 <sup>T</sup> | 37.9                | 15.1  | 10.4  |

\*Determined by HPLC.

**Table 3.** Characteristics differentiating *Bacillus krulwichiae* from other alkaliphilic *Bacillus* spp.

1, *Bacillus krulwichiae*; 2, *Bacillus alcalophilus*; 3, *Bacillus pseudalcaliphilus*; 4, *Bacillus pseudofirmus*; 5, *Bacillus halodurans*; 6, *Bacillus clausii*; 7, *Bacillus gibsonii*. +, Positive; v, variable among strains; –, negative. Characteristics of other alkaliphilic *Bacillus* spp. are from Nielsen *et al.* (1995).

| Characteristic               | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------------------------------|---|---|---|---|---|---|---|
| Hydrolysis of:               |   |   |   |   |   |   |   |
| Casein                       | v | + | + | + | + | + | + |
| Gelatin                      | v | + | + | + | + | + | + |
| Starch                       | + | + | + | + | + | + | – |
| Tween 20                     | + | v | – | – | v | – | – |
| Tween 40                     | + | + | v | + | + | – | v |
| Tween 60                     | + | + | v | + | + | – | v |
| Deamination of phenylalanine | – | – | – | + | – | – | – |
| Reduction of nitrate         | + | v | – | – | v | + | v |
| Growth at:                   |   |   |   |   |   |   |   |
| 10 °C                        | – | + | + | + | – | – | v |
| 40 °C                        | + | + | + | + | + | + | – |
| 50 °C                        | – | – | – | – | + | + | – |
| pH 7                         | – | – | – | v | v | + | + |

Type strain is AM31D<sup>T</sup> (= NCIMB 13904<sup>T</sup> = JCM 11691<sup>T</sup> = IAM 15000<sup>T</sup>).

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