Bacillus trypoxylicola sp. nov., xylanase-producing alkaliphilic bacteria isolated from the guts of Japanese horned beetle larvae (*Trypoxylus dichotomus septentrionalis*)

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Three xylanase-producing alkaliphilic strains, SU1^T, 36AC4 and 36AC6, were isolated from the guts of larvae of the Japanese horned beetle (*Trypoxylus dichotomus septentrionalis*). The isolates stained Gram-positive and were aerobic, spore-forming, non-motile and rod-shaped and grew optimally at 30 °C and pH 9. They contained MK-7 as the major isoprenoid quinone and iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{17:0} as the major fatty acids. The DNA G + C contents of the strains were 37.4–37.7 mol%. On the basis of 16S rRNA gene sequence similarity, these strains were shown to belong to the genus *Bacillus*. Although their 16S rRNA gene sequence similarity to the type strains of the alkaliphilic species *Bacillus pseudocaliphilus* and *B. alcalophilus* was 97 %, the novel isolates formed a distinct group in the phylogenetic trees and DNA–DNA relatedness values to the type strains of these species were less than 30 %. Results of physiological and biochemical tests, including salt preference, enabled these strains to be differentiated phenotypically from described *Bacillus* species. Therefore, strains SU1^T, 36AC4 and 36AC6 represent a novel species for which the name *Bacillus trypoxylicola* sp. nov. is proposed; the type strain is SU1^T (=NBRC 102646^T =KCTC 13244^T).

Since the first report on alkali-active enzymes produced by alkaliphilic strains of the genus *Bacillus*, these bacteria have been studied extensively from an industrial point of view (Horikoshi, 1991, 1996; Nielsen *et al.*, 1995; Yumoto *et al.*, 1998) and there have been many reports of alkaliphilic or alkalitolerant species of *Bacillus*, including *Bacillus agar-adhaerens*, *B. alcalophilus*, *B. clarkii*, *B. clausii*, *B. gibsonii*, *B. halmaphalus*, *B. halodurans*, *B. horikoshii*, *B. pseudocaliphilus* and *B. pseudofirmus* (Nielsen *et al.*, 1995), *B. okuhidensis* (Li *et al.*, 2002), *B. krulwichiae* (Yumoto *et al.*, 2003), *B. patagoniensis* (Olivera *et al.*, 2005), *B. oshimensis* (Yumoto

et al., 2005), *B. bogoriensis* (Vargas *et al.*, 2005), *B. akibai*, *B. cellulosityticus*, *B. hemicellulosityticus*, *B. mannanilyticus* and *B. wakoensis* (Nogi *et al.*, 2005), *B. okhensis* (Nowlan *et al.*, 2006), *B. lehensis* (Ghosh *et al.*, 2007), *B. qingdaonensis* (Wang *et al.*, 2007), *B. murimartini* and *B. plakortidis* (Borchert *et al.*, 2007), *B. polygoni* (Aino *et al.*, 2008), *B. alkalinitrilicus* (Sorokin *et al.*, 2008a, 2009), *B. alkalidiazotrophicus* (Sorokin *et al.*, 2008b), *B. alkalitelluris* (Lee *et al.*, 2008) and *B. aurantiacus* (Borsodi *et al.*, 2008). Larvae of the Japanese horned beetle, *Trypoxylus* (*Allomyrina*) *dichotomus* (Kono, 1931; Kurosawa, 1985), feed on decayed wood or leaf litter, but not fresh wood, and are assumed to play an important role in the degradation of polysaccharides produced by broadleaf trees in forests (Supplementary Fig. S1, available in IJSEM Online). It has been reported that the contents of the larval midgut have an alkaline pH (pH 10) and strong β -1,4-xylan-degrading activity (Wada *et al.*, 2004). During screening for xylanolytic alkaliphiles from the guts of larvae of *T. dichotomus* subsp. *septentrionalis* (Takeishi *et al.*, 2006), three strains, designated SU1^T, 36AC4 and 36AC6, were isolated. Based on the results of phenotypic, genotypic, chemotaxonomic and

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SU1^T, 36AC4 and 36AC6 are AB434284, AB482197 and AB482198, respectively.

A photograph of a Japanese horned beetle larva, graphs showing the effects of sodium and potassium on growth of *B. trypoxylicola* strains and related strains and fatty acid compositions of *B. trypoxylicola* strains and related strains are available as supplementary material with the online version of this paper.

phylogenetic analyses made in the present study, these strains were affiliated with the genus *Bacillus*; data obtained also suggest that the isolates represent a novel species of the genus *Bacillus*.

After having been enriched three times by being cultured in XA liquid medium [containing (l^{-1}): 5 g xylan (Sigma), 200 mg polypeptone, 200 mg Bacto yeast extract, 200 mg $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4 and 10 g K_2CO_3 (pH 10) (Takeishi *et al.*, 2006)], strain SU1^T was isolated from the gut of a larva on XA agar plates (solidified with 15 g agar l^{-1}) at 30 °C under aerobic conditions. Strains 36AC4 and 36AC6 were both isolated from the midgut of a different larva at 30 °C under aerobic conditions by using a medium of the same composition as XA agar except that xylan was replaced by 0.5 % glucose. The three strains formed yellowish, flat, round colonies with entire margins on XA agar plates. On these plates, the strains formed a clear zone around each colony, indicating the presence of xylanase activity. The strains showed good growth in alkaline tryptic soy medium [ATSM, containing (l^{-1}) 30 g Bacto tryptic soy broth (Difco) and 10 g K_2CO_3 (pH 9)] at 17–37 °C, with optimum growth at 30 °C, but did not grow at 40 °C. The effect of pH on growth was determined as described previously (Nielsen *et al.*, 1995). Strains SU1^T, 36AC4 and 36AC6 showed good growth at pH 8–10, with optimum growth at pH 9, but little or no growth below pH 7 or above pH 11 when cultured at 30 °C for 5 days. The strains showed good growth on ATS agar (ATSM solidified by the addition of 15 g agar l^{-1} ; pH 9) under aerobic conditions, but not under a nitrogen atmosphere of 99.99 %, suggesting that they are aerobic. The presence of spores was determined by the Schaeffer–Fulton staining method (Smibert & Krieg, 1994) as well as by phase-contrast microscopy. Spores were terminal in position and ellipsoidal; slight swelling of the sporangia was observed. Cells of strains SU1^T, 36AC4 and 36AC6 stained Gram-positive (Ryu, 1938) and showed no motility. Morphology was observed by phase-contrast microscopy. Cells cultured on

XA agar plates at 30 °C for 2 days were irregular rods, 0.6 µm wide and 0.8–2.0 µm long.

The 16S rRNA genes of the strains were amplified by PCR using universal primers (Tamura & Hatano, 2001) and nearly complete 16S rRNA gene sequences (1509 bp) were determined. Sequence match analysis using the Ribosome Database Project II revealed that the 16S rRNA gene sequences of strains SU1^T, 36AC4 and 36AC6 showed higher similarities to sequences of the type strains of alkaliphilic *Bacillus* species than to those of other *Bacillus* species. 16S rRNA gene sequence similarities of strain SU1^T to strain 36AC4, strain 36AC6, *B. pseudocaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T were 99.3, 99.5, 97.4 and 97.0 %, respectively. Phylogenetic relationships with closely related species were determined by using MEGA version 4 (Tamura *et al.*, 2007) and the PHYLIP 3.65 package (Felsenstein, 2005) after multiple alignments of the data performed with CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were computed as described previously (Jukes & Cantor, 1969). Phylogenetic trees were constructed by using the maximum-parsimony (Kluge & Farris, 1969), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. The reliabilities of these tree topologies were evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). Phylogenetic trees constructed by these three methods were similar topologically and showed that strains SU1^T, 36AC4 and 36AC6 belonged to the genus *Bacillus*. The phylogenetic tree constructed using the neighbour-joining method is shown in Fig. 1. Although the strains showed high degrees of 16S rRNA gene sequence similarity to described species of the genus, they formed a separate line of descent in the phylogenetic cluster of the genus. Stackebrandt & Goebel (1994) have pointed out that a high degree of 16S rRNA gene sequence similarity (≥ 97 %) is of limited value for differentiating species and that DNA–DNA hybridization studies need to be performed to determine species affiliation under these circumstances. Therefore, DNA–

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (positions 58–1440 of the *Escherichia coli* 16S rRNA gene) showing the position of strains SU1^T, 36AC4 and 36AC6 among their phylogenetic neighbours. Numbers at branch nodes are percentages based on 1000 resamplings; only values over 50 % are given. The sequence of *Bacillus pseudofirmus* DSM 8715^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

DNA hybridization studies of the novel strains and closely related strains from the phylogenetic tree were performed by using the microplate hybridization method (Ezaki *et al.*, 1988, 1989; Tamura *et al.*, 1999). The DNA–DNA relatedness values of strain SU1^T with strains 36AC4 and 36AC6 were 80.9 and 85.8 %, respectively, which were higher than the recommended threshold value for the delineation of genomic species (70 %; Wayne *et al.*, 1987). Strain SU1^T exhibited relatively low levels of DNA–DNA relatedness to *B. pseudalcaliphilus* CIP 105304^T (26.4 %) and *B. alcalophilus* NBRC 15653^T (19.6 %), indicating that it was not related to them at the species level. These data suggest that strains SU1^T, 36AC4 and 36AC6 represent a novel species of the genus *Bacillus*.

The major isoprenoid quinone of the strains was MK-7, determined using a Quattro premier mass spectrometer coupled to an ACUITY UPLC (UPLC/MS/MS; Waters). The DNA G+C content was determined as described previously (Tamaoka & Komagata, 1984). The DNA G+C contents of SU1^T, 36AC4, 36AC6, *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T were 37.4, 37.5, 37.7, 39.0 and 37.4 mol%, respectively. The isolates and the type strains of closely related species were cultivated on ATS agar (pH 9.0) for 3 days at 30 °C; cellular fatty acids were then saponified, methylated and extracted as described previously (Tamura *et al.*, 1994). Fatty acid methyl esters were analysed by using a Shimadzu QP5050 GC-MS equipped with an SPB-1 column (Supelco). The fatty acid compositions of strains SU1^T, 36AC4 and 36AC6 were similar, containing iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{17:0} as the major fatty acids (Supplementary Table S1). Fatty acid compositions were similar to those of *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T except for the relative amounts of iso-C_{16:0} and iso-C_{17:1}. The isomer type of diamino acid of the cell-wall peptidoglycan was determined as described previously (Komagata & Suzuki, 1987). Strains SU1^T, 36AC4 and 36AC6 contained *meso*-diaminopimelic acid as the diagnostic diamino acid. Standard physiological tests were carried out as described previously (Smibert & Krieg, 1994). Enzyme activity was assessed by using the API 20E, API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions (incubation times of up to 7 h). The phenylalanine deamination test was performed as described by Gordon *et al.* (1973). Acid production from carbohydrates and utilization of various substrates as sole carbon sources were tested by using the API 50CH system with the medium described by Hugh & Leifson (1953) with a modification for alkaliphiles (Yumoto *et al.*, 2003). Resistance to antibiotics was examined by using the API VET system (bioMérieux) as described by the manufacturer. Strains SU1^T, 36AC4 and 36AC6 could be distinguished from the two related alkaliphilic *Bacillus* species, *B. pseudalcaliphilus* and *B. alcalophilus*, on the basis of physiological characteristics (Table 1). In addition, growth of the isolates in the presence of sodium or potassium salts differed from that

observed with *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T. Strains SU1^T, 36AC4 and 36AC6 showed good growth (OD₆₆₀=1.2–1.3) in tryptic soy medium (Difco) adjusted to pH 9 with KOH compared with growth observed in the medium adjusted to pH 9 with NaOH (OD₆₆₀=0.5–0.6), whereas *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T showed good growth on medium adjusted with either hydroxide (OD₆₆₀=1.2–1.5). The effects of NaCl or KCl on the growth of SU1^T, 36AC4, 36AC6, *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T were determined on ATSM containing various concentrations of NaCl or KCl (Supplementary Fig. S2). Strains SU1^T, 36AC4, and 36AC6 showed a preference for K⁺ over Na⁺ in the alkaline medium, i.e. they showed good growth up to 12 % (w/v) KCl, but little or no growth in the presence of 7 % (w/v) NaCl or above (Supplementary Fig. S2a–c). Growth of *B. pseudalcaliphilus* CIP 105304^T and *B. alcalophilus* NBRC 15653^T in the presence of KCl or NaCl did not show such big differences (Supplementary Fig. S2d, e), and the former showed higher tolerance to both salts than the latter. A similar preference for K⁺ over Na⁺ in alkaline medium has been reported for alkaliphiles isolated from soil (Kitada & Horikoshi, 1987) and the alkaline gut of soil-feeding termites (Ohkuma *et al.*, 2003; Thongaram *et al.*, 2003) and *T. dichotomus* (Takeishi *et al.*, 2006).

Therefore, based on the phenotypic and phylogenetic characterization described above, strains SU1^T, 36AC4, and 36AC6 represent a novel species, for which the name *Bacillus trypoxylicola* sp. nov. is proposed.

Description of *Bacillus trypoxylicola* sp. nov.

Bacillus trypoxylicola [try.po.xy.li'co.la. N.L. masc. n. *Trypoxylus* systematic genus name of the Japanese horned beetle (Coleoptera: Scarabaeidae), the source of the type strain; L. suff. -cola an inhabitant of a place, a resident; N.L. masc. n. *trypoxylicola* the Japanese horned beetle dweller].

Gram-stain-positive, aerobic, non-motile and rod-shaped (0.6 µm wide and 0.8 to 2.0 µm long). Spores are terminal in position and ellipsoidal; slightly swollen sporangia are observed. Colonies are yellowish, flat and round with entire margins after 3 days cultivation at 30 °C on ATS agar (pH 9.0). Growth occurs between 17 and 37 °C; optimum growth at 30 °C. The pH range for growth is 8–10, with optimum growth at pH 9. Growth occurs at higher concentrations of KCl (0–17 %, w/v) than of NaCl (0–5 %, w/v). Positive for hydrolysis of xylan, but negative for hydrolysis of gelatin, casein and Tweens 20, 40, 60 and 80. Positive for oxidase, acid and alkaline phosphatases, catalase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, naphthol-AS-BI-phosphohydrolase and leucine, trypsin and valine arylamidases, but negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-mannosidase, lipase (C4), lysine and ornithine decarboxylases, tryptophan deaminase, urease,

Table 1. Physiological characteristics of strains of *Bacillus trypoxylicola* sp. nov. and closely related *Bacillus* type strains

Strains: 1, *B. trypoxylicola* sp. nov. SU1^T, 36AC4 and 36AC6; 2, *B. pseudocaliphilus* CIP 105304^T; 3, *B. alcalophilus* NBRC 15653^T. Data were obtained in this study. All strains had the following characteristics: positive for Gram staining, oxidase and catalase; positive for alkaline phosphatase, esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and α -glucosidase (API ZYM); negative for hydrolysis of Tween 80 and deamination of phenylalanine; negative for lipase (C4), *N*-acetyl-D-glucosaminidase, α -mannosidase, α -fucosidase, cystine arylamidase, Voges–Proskauer test, lysine and ornithine decarboxylases, tryptophan deaminase, indole production, hydrogen sulfide production, urease, proteinase, arginine dihydrolase and reduction of nitrate and nitrite (API ZYM, API 20E and API 20NE); positive for acid production from D-glucose, D-fructose, maltose, sucrose, trehalose, starch and glycogen (API 50CH); negative for acid production from erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, arbutin, melezitose, D-lyxose, D-fucose, D- and L-arabitol, potassium gluconate and potassium 5-ketogluconate (API 50CH); positive for utilization of glycerol, methyl β -D-xylopyranoside, D-galactose, D-glucose, D-fructose, L-rhamnose, D-mannitol, methyl α -D-glucopyranoside, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, starch and glycogen (API 50CH); negative for utilization of erythritol, L-xylose, D-adonitol, L-sorbose, dulcitol, D-sorbitol, inulin, melezitose, xylitol, D- and L-arabitol, potassium gluconate and potassium 5-ketogluconate (API 50CH); resistant to metronidazole and nitrofurantoin (API VET); and sensitive to amoxicillin, augmentin, cephalothin, chloramphenicol, doxycycline, gentamicin, penicillin, oxacillin and tetracycline (API VET). +, Positive; –, negative; (+), weakly positive.

Characteristic	1	2	3
Growth in:			
7 % (w/v) NaCl	–	+	+
10 % (w/v) NaCl	–	+	–
12 % (w/v) KCl	+	+	–
Hydrolysis of:			
Tween 20	–	–	(+)
Tweens 40 and 60	–	+	+
Casein	–	+	+
Gelatin	–	+	+
Activity of (API ZYM):			
Esterase (C4)	+	+	–
Leucine arylamidase	+	–	+
Valine arylamidase	+	–	–
Trypsin	+	–	–
α -Chymotrypsin	–	+	+
Acid phosphatase	+	+	–
α -Galactosidase	+	–	–
β -Galactosidase	+	–	+
β -Glucuronidase	+	–	–
β -Glucosidase	+	–	–
Acid production from (API 50CH):			
L-Arabinose	+	–	+
D-Ribose	(+)	–	+
D-Xylose	+	–	+
D-Galactose	+	–	–
D-Mannose	+	–	+

Table 1. cont.

Characteristic	1	2	3
L-Rhamnose	+	+	–
D-Mannitol	+	–	–
D-Sorbitol	(+)	–	+
Methyl α -D-mannopyranoside	–	–	+
Methyl α -D-glucopyranoside	–	+	+
<i>N</i> -Acetylglucosamine	–	–	+
Cellobiose	+	–	–
Lactose	+	–	+
Melibiose	+	–	+
Inulin	–	–	+
Raffinose	+	–	+
D-Tagatose	–	–	+
Potassium 5-ketogluconate	–	–	(+)
Utilization of (API 50CH):			
D-Arabinose	–	–	(+)
D-Ribose	–	+	+
D-Mannose	+	–	+
Inositol	–	–	(+)
Methyl α -D-mannopyranoside	+	–	–
<i>N</i> -Acetylglucosamine	–	–	+
Melibiose	+	–	+
Raffinose	+	–	+
Turanose	–	+	+
D-Lyxose	–	+	+
D-Tagatose	–	+	+
Potassium 2-ketogluconate	+	–	–
Resistance to (API VET):			
Streptomycin	–	+	–
Kanamycin	+	(+)	–
Erythromycin	–	+	+
Lincomycin	–	+	–
Colistin	–	+	+
Sulfamethizole	+	–	+
Flumequine	+	–	(+)
Oxolinic acid	+	–	(+)
Rifampicin	–	+	–

arginine dihydrolase and proteinase (API ZYM, API 20E and API 20NE systems). Negative for the Voges–Proskauer test, indole production, hydrogen sulfide production and reduction of nitrate and nitrite (API 20E and API 20NE systems). Negative for the phenylalanine deamination test. Positive for acid production from L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch and glycogen, but negative for acid production from erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, *N*-acetylglucosamine, arbutin, inulin, melezitose, D-lyxose, D-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate (API 50CH system). Positive for utilization of glycerol, methyl β -D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol,

methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, arbutin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, raffinose, starch, glycogen and potassium 2-ketogluconate, but negative for utilization of erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, D-sorbitol, N-acetylglucosamine, inulin, melezitose, xylitol, turanose, D-lyxose, D-tagatose, D- and L-arabitol, potassium gluconate and potassium 5-ketogluconate (API 50CH system). Resistant to flumequine, kanamycin, metronidazole, nitrofurantoin, oxolinic acid and sulfamethizole, but sensitive to amoxicillin, apramycin, augmentin, cefoperazone, cephalothin, chloramphenicol, colistin, doxycycline, erythromycin, gentamicin, lincomycin, oxacillin, penicillin, pristnamycin, rifampicin, streptomycin, spectinomycin, tetracycline and tylosin (API VET system). The major isoprenoid quinone is MK-7, the cell-wall peptidoglycan contains *meso*-diaminopimelic acid, and the major fatty acids (>10% of total fatty acids) are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{17:0}.

The type strain is SU1^T (=NBRC 102646^T =KCTC 13244^T), isolated from the gut of a Japanese horned beetle larva (*Trypoxylus dichotomus septentrionalis*). The DNA G+C content of the type strain is 37.4 mol%.

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