Anoxybacillus tengchongensis sp. nov. and *Anoxybacillus eryuanensis* sp. nov., facultatively anaerobic, alkalitolerant bacteria from hot springs

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Two novel thermophilic, spore-forming bacterial strains, T-11^T and E-112^T, were isolated from hot springs in Tengchong and Eryuan counties of Yunnan province in south-west China. The strains were Gram-stain-positive rods, occurring singly or in chains. Growth of strain T-11^T was observed between 30 and 75 °C (optimum 50 °C) and at pH 7–11 (optimum pH 8.5), while the temperature range for strain E-112^T was 35–70 °C (optimum 55 °C) and the pH range was 7.0–11.0 (optimum pH 8.0). The DNA G+C contents of strains T-11^T and E-112^T were 41.1 and 42.6 mol%, respectively. On the basis of 16S rRNA gene sequence similarity, the two strains were shown to be related most closely to *Anoxybacillus* species. The chemotaxonomic characteristics [predominant isoprenoid quinone menaquinone 7 (MK-7); major fatty acids iso-$C_{15:0}$ and iso-$C_{17:0}$] also supported the affiliation of strains T-11^T and E-112^T to the genus *Anoxybacillus*. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strains T-11^T and E-112^T from *Anoxybacillus* species with validly published names. Strains T-11^T and E-112^T therefore represent two novel species, for which the names *Anoxybacillus tengchongensis* sp. nov. (type strain T-11^T =CCTCC AB209237^T =KCTC 13721^T) and *Anoxybacillus eryuanensis* sp. nov. (type strain E-112^T =CCTCC AB209236^T =KCTC 13720^T) are proposed.

The first representative of the genus *Anoxybacillus*, *Anoxybacillus pushchinoensis*, was described by Pikuta *et al.* (2000) as strictly anaerobic. However, according to the emended description of this species published later (Pikuta *et al.*, 2003), it is facultatively aerobic. Further representatives of the genus *Anoxybacillus* have subsequently been reported and at the time of writing the genus includes *A. pushchinoensis* and *A. flavithermus* (Pikuta *et al.*, 2000), *A. gonensis* (Belduz *et al.*, 2003), *A. contaminans* (De Clerck *et al.*, 2004), *A. ayderensis* and *A. kestanbolensis* (Dulger *et al.*, 2004), *A. voinovskiensis* (Yumoto *et al.*, 2004), *A. kamchatkensis* (Kevbrin *et al.*, 2005), *A. amylolyticus* (Poli *et al.*, 2006), *A. rupiensis* (Derekova *et al.*, 2007) and *A. bogrovensis* (Atanassova *et al.*, 2008). Although the name of the genus *Anoxybacillus* means 'bacillus that does not require oxygen' (Pikuta *et al.*, 2000), most of the described species can grow well aerobically and, for some species, anaerobic growth is registered only under certain conditions (Yumoto *et al.*, 2004).

Here, we describe two novel thermophilic bacteria isolated from the areas of Tengchong and Eryuan in Yunnan province, China. The two bacterial strains were isolated from water samples collected at hot springs with temperatures of around 50 °C. After collection, the water samples were used immediately for enrichment in LB medium (1 % tryptone, 1 % NaCl, 0.5 % yeast extract) at 50 °C. One-day-old enrichment cultures were repeatedly subcultured in 10 ml LB and streaked on agar plates to separate colonies. The purity of the cultures was assessed by colony morphology and microscopy. Growth was determined by measuring changes in OD_{600}. Gram staining was carried out by standard procedures using cultures grown overnight on LB agar.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains T-11^T and E-112^T are FJ438370 and GQ153549, respectively.

Cells of strains T-11^T and E-112^T appeared as Gram-staining-positive, motile, round-ended rods. Light microscopy revealed that cells of strain T-11^T were 0.6–1.2 × 4.5–5.5 μm and cells of strain E-112 ^{T} were 0.5–0.7 × 4.5–4.7 μm. In the exponential growth phase, most cells of both strains occurred singly or in chains. Colonies of strain T-11^T were 1–2.5 mm in diameter, usually cream, smooth and circular with round edges after 12 h growth. Colonies of strain E-112^T were the same except that they were 1–2 mm in diameter under the same conditions. Light microscopy revealed that strains T-11^T and E-112^T were sporulating bacilli with ellipsoidal or cylindrical terminal endospores. The formation of spores was observed using samples either from liquid cultures or single colonies on agar plates. Incubation periods of 1–2 days were required before spore formation became detectable. Both strains were determined to be facultative aerobes according to the method of Schäffer *et al.* (2004).

The effects of pH on growth were determined over the range pH 7.0–12.0 at intervals of 0.5 pH units using HCl and NaOH to adjust the pH after cultures had been incubated overnight. The influence of temperature on growth was assayed at pH$^{25\ ^\circ C}$ 8.5 at 5 °C intervals (20–80 °C) with shaking overnight. Strain T-11^T grew well at 30–75 °C, with optimal growth at 50 °C, and at pH$^{25\ ^\circ C}$ 7.0–11.0, with optimum growth at pH 8.5. For strain E-112^T, the temperature range for growth was 35–70 °C (optimal temperature 55 °C) and the pH$^{25\ ^\circ C}$ range was pH 7.0–11.0 (optimum pH 8.0). The shortest doubling times for strains T-11^T and E-112^T were 30 and 38 min, respectively, under optimal conditions for growth. Strains T-11^T and E-112^T were catalase- and oxidase-positive and both strains were able to utilize a broad spectrum of carbohydrates such as sugars, polysaccharides and polyols in the presence of proteinaceous substrates or inorganic nitrogen. Growth of strain T-11^T was observed on glucose, trehalose, D-mannose, sucrose, D-fructose, raffinose, maltose and D-mannitol, but not on xylose, lactose or L-rhamnose. It was positive for gelatin hydrolysis and starch hydrolysis and could reduce nitrate. Strain E-112^T could also utilize a wide range of carbon sources including glucose, trehalose, mannitol, sucrose, D-fructose, maltose and mannose, but not xylose, lactose, raffinose or L-rhamnose. It was positive for gelatin hydrolysis and starch hydrolysis, but could not reduce nitrate (Table 1). Growth of strains T-11^T and E-112^T was inhibited in the presence of NaCl concentrations above 4.0 and 3.0 %, respectively; however, the optimal NaCl concentrations for growth of T-11^T and E-112^T were 1.5 and 0.5 %. Sensitivity to ampicillin, tetracycline, gentamicin, streptomycin, carbenicillin, chloramphenicol (each at 10 μg ml^{-1}) was tested. The cultures were incubated at 50 °C and pH$^{25\ ^\circ C}$ 8.5. Strain T-11^T was sensitive to chloramphenicol, but insensitive to ampicillin, tetracycline, gentamicin, streptomycin and carbenicillin. Strain E-112^T was sensitive to chloramphenicol and ampicillin, but insensitive to tetracycline, gentamicin, streptomycin and carbenicillin.

Cultivation, harvesting, preparation and analysis of cellular fatty acid methyl esters from total fatty acids were performed according to the method described in the Sherlock Microbial Identification System manual (version 4.0; MIDI). The fatty acid profiles of strains T-11^T and E-112^T were composed largely of branched saturated fatty acids with minor amounts of anteiso-fatty acids, as for most other *Anoxybacillus* species (Table 2); for members of the genus *Anoxybacillus*, iso-branched saturated fatty acids (iso-$C_{15:0}$ and iso-$C_{17:0}$) are dominant. In our results, the major cellular fatty acids for T-11^T were also iso-$C_{15:0}$ (60.5 %) and iso-$C_{17:0}$ (7.0 %), with smaller amounts of iso-$C_{16:0}$ (5.9 %) and anteiso-$C_{15:0}$ (2.8 %). Similarly, the major cellular fatty acids for E-112^T were iso-$C_{15:0}$ (60.7 %) and iso-$C_{17:0}$ (8.5 %), with smaller amounts of anteiso-$C_{15:0}$ (3.5 %) and iso-$C_{16:0}$ (2.2 %). The isoprenoid quinone of the two strains was isolated according to Collins *et al.* (1977) and then analysed by HPLC (Groth *et al.*, 1997). Our results showed that the predominant isoprenoid quinone for both strains was MK-7; MK-6 was also detected.

Extraction of genomic DNA and amplification of the 16S rRNA gene from strains E-112^T and T-11^T were performed as described by Li *et al.* (2007). The resulting sequences were aligned manually against sequences obtained from the GenBank database using the CLUSTAL_X 1.8 program (Thompson *et al.*, 1997). Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar *et al.*, 2004). DNA was purified on hydroxyapatite based on the procedure of Cashion *et al.* (1977). The DNA G+C content was determined according to Mesbah *et al.* (1989) by using HPLC. Non-methylated lambda DNA (Sigma) (G+C content 49.858 mol%) was used as a reference. The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT). DNA–DNA relatedness experiments by the thermal denaturation method in the liquid phase were carried out as described by De Ley *et al.* (1970) with the modification described by Huß *et al.* (1983).

On the basis of 16S rRNA gene sequence analysis, isolates T-11^T and E-112^T showed high sequence similarity to members of the genus *Anoxybacillus* (Fig. 1). Strain T-11^T showed highest similarity to *A. pushchinoensis* K1^T (98.3 %), *A. flavithermus* DSM 2641^T (98.2 %), *A. kamchatkensis* JW/VK-KG4^T (97.6 %) and *A. ayderensis* AB04^T (97.4 %). Strain E-112^T showed highest similarity to *A. flavithermus* DSM 2641^T (98.3 %), *A. pushchinoensis* K1^T (98.2 %), *A. kamchatkensis* JW/VK-KG4^T (97.4 %) and *A. ayderensis* AB04^T (97.3 %). Accordingly, comparative taxonomic studies were performed among these six strains, T-11^T, E-112^T, *A. flavithermus* DSM 2641^T, *A. pushchinoensis* DSM 12423^T, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* NCIMB 13972^T. Our results revealed that strains T-11^T and E-112^T had some differences in their phenotypic characteristics from these four closest neighbours (Table 1). Additionally, DNA–DNA hybridization was performed for strains T-11^T and E-112^T with the closest type strains.

Table 1. Phenotypic characteristics of *Anoxybacillus tengchongensis* sp. nov. T-11^T, *Anoxybacillus eryuanensis* sp. nov. E-112^T and related *Anoxybacillus* type strains

Strains: 1, *A. tengchongensis* sp. nov. T-11^T; 2, *A. eryuanensis* sp. nov. E-112^T; 3, *A. pushchinoensis* DSM 12423^T (data from Pikuta *et al.*, 2000, 2003); 4, *A. flavithermus* DSM 2641^T (Heinen *et al.*, 1982); 5, *A. kamchatkensis* DSM 14988^T (Kevbrin *et al.*, 2005); 6, *A. ayderensis* NCIMB 13972^T (Dulger *et al.*, 2004). The pH was measured with a calibrated pH meter; the values given are for pH$^{25\ °C}$ (Wiegel, 1998). All strains are facultative anaerobes.

Characteristic	1	2	3	4	5	6
Temperature range (°C)	30–75	35–70	37–66	30–72	38–67	30–70
Optimum temperature (°C)	50	55	62	60–65	57–62	50
pH range	7.0–11.0	7.0–11.0	8.0–10.5	5.5–9.0	5.7–9.9	6.0–11.0
Optimum pH	8.5	8	9.5–9.7	7	6.8–8.5	7.5–8.5
Maximum NaCl concentration (%, w/v)	4	3	3	2.5	3	2.5
Motility	+	+	−	+	+	+
Hydrolysis of:						
Gelatin	+	+	−	−	−	+
Starch	+	+	+	+	−	+
Oxidase	+	+	−	+	−	+
Use as a sole carbon source of:						
Glucose	+	+	+	−	+	+
Raffinose	−	−	−	−	−	+
Nitrate reduction	+	−	−	+	+	+

The relatedness between strain T-11^T and *A. pushchinoensis* DSM 12423^T, *A. flavithermus* DSM 2641^T, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* NCIMB 13972^T was 30.2, 33.6, 41.7 and 60.2 %, respectively, and the relatedness between strain E-112^T and *A. pushchinoensis* DSM 12423^T, *A. flavithermus* DSM 2641^T, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* NCIMB 13972^T was 37.4, 53.4, 35.9 and 28.7 %, respectively. Furthermore, the relatedness between our two strains was 46.4 %. Taking into account the cut-off value of 70 % relatedness suggested by Stackebrandt *et al.* (2002), these results indicated that the two new isolates and the four reference type strains belonged to different species.

Table 2. Cellular fatty acid compositions of *A. tengchongensis* sp. nov. T-11^T, *A. eryuanensis* sp. nov. E-112^T and related *Anoxybacillus* type strains

Strains: 1, *A. tengchongensis* sp. nov. T-11^T; 2, *A. eryuanensis* sp. nov. E-112^T; 3, *A. ayderensis* NCIMB 13972^T; 4, *A. flavithermus* DSM 2641^T; 5, *A. kamchatkensis* DSM 14988^T; 6, *A. pushchinoensis* DSM 12423^T. Data were obtained in this study. Values are percentages by weight of total fatty acids; −, not detected.

Fatty acid	1	2	3	4	5	6
iso-$C_{14:0}$	4.0	0.8	2.9	0.3	2.5	0.7
$C_{14:0}$	1.0	1.8	1.4	2.4	1.1	1.3
iso-$C_{15:0}$	60.5	60.7	57.7	51.4	57.8	52.7
anteiso-$C_{15:0}$	2.8	3.5	2.2	4.8	1.7	3.9
$C_{15:0}$	0.3	0.3	0.3	0.4	–	0.4
iso-$C_{16:0}$	5.9	2.2	7.2	1.5	9.9	3.0
$C_{16:0}$	2.4	3.9	2.8	6.1	2.5	4.2
iso-$C_{17:0}$	7.0	8.5	7.3	9.6	7.5	14.8
$C_{17:0}$	2.1	2.4	1.5	3.8	1.3	1.1

The genomic DNA G+C contents of strains T-11^T and E-112^T were determined by HPLC as 41.1 and 42.6 mol%, respectively, which lie within the range for the genus *Anoxybacillus*. The genomic DNA G+C content of strain T-11^T was lower than those of all related type strains, including *A. flavithermus* DSM 2641^T, *A. pushchinoensis* DSM 12423^T, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* NCIMB 13972^T. Strain E-112^T had a higher DNA G+C content than *A. kamchatkensis* DSM 14988^T.

Therefore, based on the above phenotypic and genotypic results, we consider strains T-11^T and E-112^T to represent two novel species of the genus *Anoxybacillus*, for which the names *Anoxybacillus tengchongensis* sp. nov. and *Anoxybacillus eryuanensis* sp. nov. are proposed.

Description of *Anoxybacillus tengchongensis* sp. nov.

Anoxybacillus tengchongensis (teng.chong.en′sis. N.L. masc. adj. *tengchongensis* pertaining to Tengchong, Yunnan province, south-west China, where the type strain was isolated).

Cells appear as Gram-staining-positive, motile, slightly curved rods, approx. 4.5–5.5 μm long and 0.6–1.2 μm wide. In the exponential growth phase, most cells occur singly or in chains. Terminal ellipsoidal or cylindrical endospores are observed. Colonies are 1–2.5 mm in diameter, usually cream, smooth and circular with round edges. Obligate thermophile, growing between 30 and 75 °C with optimum growth around 50 °C (no growth below 30 or above 75 °C); grows at pH$^{25\ °C}$ 7.0–11.0 (optimum around pH 8.5; no growth below pH 7.0 or above pH 11.0). Facultative aerobe. Positive for oxidase, catalase and hydrolysis of starch and gelatin. Able to utilize

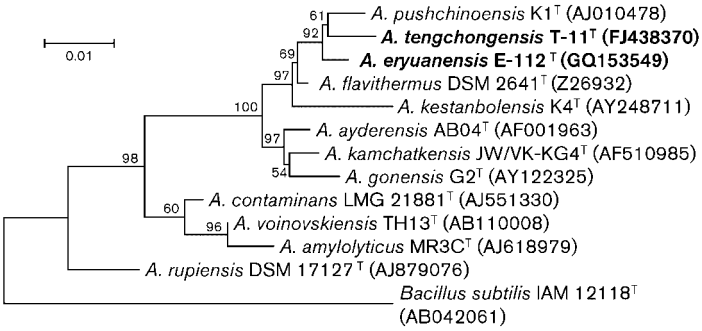

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the neighbour-joining method (Saitou & Nei, 1987) showing the positions of strains T-11^T and E-112^T among members of the genus *Anoxybacillus*. The sequence of *Bacillus subtilis* IAM 12118^T was used as an outgroup. Numbers at branching points refer to bootstrap percentages (1000 resamplings; only values above 50 % are shown). Bar, 1 substitution per 100 nucleotide positions.

a broad spectrum of carbohydrates such as sugars, polysaccharides and polyols in the presence of proteinaceous substrates or inorganic nitrogen. Grows on glucose, trehalose, D-mannose, sucrose, D-fructose, maltose and D-mannitol. Growth is not observed on xylose, lactose, raffinose or L-rhamnose. Tolerates 4.0 % NaCl; optimal NaCl concentration for growth is 1.5 %. Sensitive to chloramphenicol, but insensitive to ampicillin, tetracycline, gentamicin, streptomycin and carbenicillin. The predominant isoprenoid quinone is MK-7. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:0}$. The DNA G+C content of the type strain is 41.1 mol%.

The type strain is T-11^T (=CCTCC AB209237^T =KCTC 13721^T), which was isolated from a hot spring in Tengchong, Yunnan province, south-west China.

Description of *Anoxybacillus eryuanensis* sp. nov.

Anoxybacillus eryuanensis (er.yuan.en′sis. N.L. masc. adj. *eryuanensis* pertaining to Eryuan, Yunnan province, south-west China, where the type strain was isolated).

Colonies on LB agar have an umbonate surface and undulate edge, 1.0–2.0 mm in diameter, usually cream, glistening and smooth. Cells are Gram-staining-positive, motile, straight rods, approx. 4.5–4.7 μm long and 0.5–0.7 μm wide. Most cells occur singly or in chains with ellipsoidal to cylindrical terminal endospores. Facultative aerobe. The temperature range for growth is 35–70 °C, with optimal growth at 55 °C. The pH range is 7.0–11.0, with an optimum at pH 8.0. NaCl is tolerated at 0–3 % (w/v); the optimal NaCl concentration for growth is 0.5 %. Positive for oxidase, catalase and starch and gelatin hydrolysis; cannot reduce nitrate. Glucose, glucose, trehalose, mannitol, sucrose, D-fructose, maltose and mannose are used as sole carbon and energy sources, whereas xylose, lactose, raffinose and L-rhamnose cannot be used for growth. The fatty acid profile contains the major components iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. The G+C content of the DNA of the type strain is 42.6 mol%.

The type strain is E-112^T (=CCTCC AB209236^T =KCTC 13720^T), which was isolated from a hot spring in Eryuan, Yunnan province, south-west China.

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