Anoxybacillus calidus sp. nov., a thermophilic bacterium isolated from soil near a thermal power plant

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A novel thermophilic, Gram-stain-positive, facultatively anaerobic, endospore-forming, motile, rod-shaped bacterium, strain C161abT, was isolated from a soil sample collected near Kızılderle, Sarayköy-Buharkent power plant in Denizli. The isolate could grow at temperatures between 35 and 70 °C (optimum 55 °C), at pH 6.5–9.0 (optimum pH 8.0–8.5) and with 0–2.5 % NaCl (optimum 0.5 %, w/v). The strain formed cream-coloured, circular colonies and tolerated up to 70 mM boron. Its DNA G+C content was 37.8 mol%. The peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid. Strain C161abT contained menaquinones MK-7 (96 %) and MK-6 (4 %). The major cellular fatty acids were iso-branched fatty acids: iso-C15:0 (52.2 %) and iso-C17:0 (28.0 %), with small amounts of C16:0 (7.4 %). Phylogenetic analysis based on the 16S rRNA gene revealed 94.6–96.8 % sequence similarity with all recognized species of the genus Anoxybacillus. Strain C161abT showed the greatest sequence similarity to Anoxybacillus rupiensis DSM 17127T and Anoxybacillus voinovskiensis DSM 17075T, both had 96.8 % similarity to strain C161abT, as well as to Anoxybacillus caldiproteolyticus DSM 15730T (96.6 %). DNA–DNA hybridization revealed low levels of relatedness with the closest relatives of strain C161abT, A. rupiensis (21.2 %) and A. voinovskiensis (16.5 %). On the basis of the results obtained from phenotypic, chemotaxonomic, genomic fingerprinting, phylogenetic and hybridization analyses, the isolate is proposed to represent a novel species, Anoxybacillus calidus sp. nov. (type strain C161abT=DSM 25520=NCIMB 14851T).

Since the first description of the genus Anoxybacillus (Pikuta et al., 2000), the number of species has increased to twenty at the time of writing, and it also has three subspecies: Anoxybacillus pushchinoensis, A. flavithermus (Pikuta et al., 2000), A. gonensis (Belduz et al., 2003), A. contaminans (De Clerck et al., 2004), A. voinovskiensis (Yumoto et al., 2004), A. kstanbolensis, A. ayderensis (Dulger et al., 2004), A. kamchatkensis (Kevbrin et al., 2005), A. amylyticus (Poli et al., 2006), A. rupiensis (Derekova et al., 2007), A. bogrovensis (Atanassova et al., 2008), 'A. kamchatkensis subsp. asacharedens' (Gul-Guven et al., 2008), A. thermarum (Poli et al., 2009), A. eryuanensis, A. tengchongensis (Zhang et al., 2010), A. salavatliensis (Cihan et al., 2011), A. mongoliensis (Namsaraev et al., 2010), A. flavithermus subsp. flavithermus, A. flavithermus subsp. yunnanensis (Dai et al., 2011), A. caldiproteolyticus (Coorevits et al., 2012), A. tepidamans (Schäffer et al., 2004; Coorevits et al., 2012), A. kaynarcensis (Ihan et al., 2013) and A. vitaminiphilus (Zhang et al., 2013). Most of the species belonging to the genus Anoxybacillus represent a homogeneous group of thermophilic bacilli with high intergenic 16S rRNA gene sequence similarity values.

During a polyphasic taxonomic study (Cihan, 2013) of 53 isolates belonging to the genus Anoxybacillus, the 16S rRNA gene sequencing and phylogenetic analyses revealed a novel thermophilic and endospore-forming strain, C161abT, isolated from a soil sample in Kızılderle, Sarayköy-Buharkent thermal power plant, Denizli. This new isolate displayed less than 96.8 % sequence similarity to any of the members of the genera Anoxybacillus and Geo Bacillus examined. Therefore, the strain was chosen for further complete polyphasic taxonomic study. The current paper describes the taxonomic position of strain C161abT.
among members of the genus *Anoxybacillus* with details of phenotypic, chemotaxonomic and genetic characteristics based upon a single isolate (no other strains were available for comparison). On the basis of the presented data, it is proposed that strain C161ab\(^T\) should be classified as a representative of a novel species, *Anoxybacillus calidus* sp. nov.

Strain C161ab\(^T\) was isolated from a soil sample collected near one of the high temperature well pipelines of Kızildere, Sarayköy-Buharkent thermal power plant in Denizli province of Turkey. The Kızildere thermal power plant is the first and also one of the industrially important geothermal plants in Turkey; it has a very high water temperature from 195 to 212 °C in its reservoir and the soil is rich in boron (18–29 mg l\(^{-1}\)) as the underground geothermal water containing boron flows up to the ground. This geothermal area is located in the Aegean Region of Turkey (37° 46’ N 29° 06’ E) and the water pH was measured to be pH 8.5–8.9. To obtain the enrichment culture, 0.1 g soil was incubated at 60 °C with 250 r.p.m. shaking for 24 h in 5 ml of MI medium (*Geobacillus thermoglucosidasius* medium) containing 1 % soluble starch (pH 7.0) (Suzuki *et al.*, 1976). The turbid cultures were streaked on plates of MI medium containing 3 % agar and incubated aerobically at 60 °C for 24 h. Colonies of strain C161ab\(^T\) were purified from the same medium by subculturing four times and the purity was also confirmed microscopically. For long-term maintenance, a turbid culture in MI broth was supplemented with 20 % (w/v) glycerol and stored at ~80 °C.

The temperature range for growth was determined by incubating the strain in nutrient broth (Merck 1.05443) medium at temperatures from 30 to 80 °C. The pH dependence was tested in nutrient broth which was adjusted with NaOH or HCl to various pH values from pH 5.0 to 11.0. The effect of salinity was also checked in nutrient broth supplemented with 0 to 5 % (w/v) NaCl. The optimal growth at different temperatures, pH and salinity was determined spectrophotometrically by measuring the OD at 600 nm. Cytology and colony morphologies were described during aerobic growth with optimal growth parameters. Colony characteristics were determined after 18 to 72 h of growth on nutrient agar or MI plates. Spore-formation was tested on nutrient agar supplemented with 5 mg MnSO\(_4\) · 4H\(_2\)O l\(^{-1}\) for 18–72 h and observed using phase-contrast microscopy (Olympus BX51) as in the case of motility tests. The morphology of cells was also observed by using a phase-contrast microscope (cells grown in nutrient broth for 6, 18, 24 and 72 h and 7 days).

Physiological characterization tests were carried out by the methods of Claus & Berkeley (1986) described previously and monitored for 1–7 days; they included Gram staining, anaerobic growth, oxidation and catalase activity, casein, citrate, starch, tyrosine, gelatin and urea utilization, reduction of nitrate to nitrite, N\(_2\) gas production from nitrate, acid production from sugars (0.5 % maltose, D-fructose, glucose, lactose, D-(+)-galactose, sucrose, D-(+)-mannose, D-(+)-xylose, D-sorbitol, L-arabinose, D-(−)mannitol, trehalose, raffinose and ribose), gas production from glucose, methyl red test, Voges–Proskauer test (pH 6.9), and indole and H\(_2\)S production. A disc diffusion test was performed on nutrient agar plates for antimicrobial susceptibility. Plasmid DNA isolation was carried out by the procedure of Anderson & McKay (1983).

Boron tolerance was tested on a culture grown in nutrient broth (pH 8.0) supplemented with different boron levels ranging from 5 mM to 450 mM boric acid and incubation was carried out at 55 °C by vigorous shaking in test tubes. Nutrient broth medium without boron was used as a control. After pre-incubation on nutrient agar for 18 h, the cell concentration was adjusted to OD\(_{600}\) 0.2–0.4 before inoculation of the cells into boron-containing broth. The MIC of boron on cells was evaluated spectrophotometrically at 600 nm, after incubation of cells for three days at 55 °C (Raja & Omine, 2012). All assays were performed with pure cultures in triplicate.

When determining the extracellular enzyme activities, the supernatants of the cultures, grown in the enzyme production media, were used for the enzyme source (Coleri *et al.*, 2009). For \(x\)-glucosidase and \(x\)-amylase assays, strains were incubated in 1 % starch-containing MI medium for 24 h, and the enzyme activities were measured by using spectrophotometric para-nitrophenol \(x\)-D-glucoside and dinitrosalicilic acid (DNS) methods, respectively (Halvorson, 1966; Coleri *et al.*, 2009). An olive oil-containing medium described by Castro-Ochoa *et al.* (2005) was used when determining the lipase activity of the 72-hour-old cultures by using para-nitrophenol butyrate as substrate (Lee *et al.*, 1999). In the protease assay, strains were cultured in a skimmed-milk-containing modified medium of Denizci *et al.* (2004) for 96 h before measuring the activity in Tris/HCl buffer (pH 7.0) by the spectrophotometric method described by Gessesse & Gashe (1997).

Fatty acid and respiratory lipoquinone analyses were carried out using minor modifications of the extraction method of Kuykendall *et al.* (1988), Miller (1982) and Tindall (1990a, b). Strains for these analyses were grown at 55 °C in TSBA40 medium (MIDI, 2002). Fatty acids were further determined using GC, whereas quinones were analysed by HPLC. All the fatty acid, quinone and cell wall composition analyses were determined at the DSMZ Identification Service, Braunschweig, Germany. In *mesodiaminopimelic* acid (meso-Dpm) content analysis, the whole cells of the strain were hydrolysed with 4 M HCl at 100 °C for 16 h and subjected to TLC on cellulose plates using the solvent system of Ruhlend *et al.* (1955).

DNA base composition and DNA–DNA hybridization were carried out by the DSMZ Identification Service. DNA was extracted using the French press technique, and purified by chromatography on hydroxyapatite by using the method of Cashion *et al.* (1977). When determining G + C base composition of the genomic DNA, the DNA was hydrolysed with P1 nuclease and the nucleotides were deporphorylated with
bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed using HPLC. The G+C content of the genomic DNA was calculated according to the method of Mesbah et al. (1989). Spectroscopic DNA–DNA hybridization was carried out at 66 °C in 2 × SSC as described by De Ley et al. (1970) with the modifications defined by Huss et al. (1983).

Cultures growing on MI plates for 18 h at 55 °C were used for genomic DNA extraction (Genomic DNA purification kit, SM1353; Fermentas). The gene encoding the 16S rRNA was amplified by PCR with the bacterial 16S rRNA gene-specific primers 27F forward and 1492R reverse (Lane, 1991). The sequence of the PCR-amplified 16S RNA gene was determined by using an ABI 3100 gene sequencer with a Bigdye cycle sequencing kit (Applied Biosystems). The gene encoding the 16S rRNA was amplified by PCR with the bacterial 16S rRNA gene-specific primers 27F forward and 1492R reverse (Lane, 1991). The sequence of the PCR-amplified 16S RNA gene was determined by using an ABI 3100 gene sequencer with a Bigdye cycle sequencing kit (Applied Biosystems). In the phylogenetic analysis, a similarity search was carried out using the basic BLASTN search program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the web-based public EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Phylogenetic analyses were performed using both the maximum-likelihood and neighbour-joining methods with bootstrap values based on 1000 replications (Felsenstein, 1985; Saitou & Nei, 1987). As the phylogenetic analyses derived from the neighbour-joining method were in congruence with those obtained using the maximum-likelihood algorithm, only the data obtained from the neighbour-joining method is presented, which was reconstructed with the MEGA package version 5 (Tamura et al., 2007). The nucleotide substitution distances were estimated by using the Jukes–Cantor model (Jukes & Cantor, 1969).

For genomic fingerprinting analyses of the repetitive extragenic palindromic (rep) elements, PCR was performed with the (GTG)₅ and BOXA1R primers using the conditions described by Versalovic et al. (1994). Primer sets S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 were used for the amplification of intergenic transcribed spacers (ITS) between the 16S and 23S rRNA genes and PCR conditions were adjusted according to Daffonchio et al. (2003). The PCR products were electrophoresed in a 1.5 % agarose gel with 1 × Tris/borate/EDTA (TBE) buffer at 120 V for 4 h. The resulting BOX-, (GTG)₅- and ITS-PCR fingerprints were analysed by the GelCompar II software package, for the presence or absence of the DNA bands, their sizes and also their densities (Applied Maths). Similarities of the digitized profiles including these three tests were calculated together using the Pearson correlation and an average linkage (UPGMA) dendrogram was obtained.

The detailed physiological characteristics of strain C161abᵀ are given in the species description. The strain differed from related species of the genus *Anoxybacillus* by means of some phenotypic characteristics as presented in Table 1. Strain C161abᵀ was catalase-negative like *A. kamchatkensis*, but gave a positive reaction for oxidase. The strain was facultatively anaerobic that differentiated strain C161abᵀ from its strictly aerobic relative: *A. rupiensis*. It was thermophilic with growth at 35–70 °C, optimal at 55 °C. The strain was alkaliphilic as it grew between pH 6.5 and 9.0, with optimal growth at pH 8.0–8.5. The NaCl tolerance was limited to 2.5 % (w/v), with an optimal concentration of 0.5 % (w/v). Since the place of isolation contained a high amount of boron, the boron tolerance was also tested. It was found that the strain required at least 5 mM boron for growth and was able to tolerate up to 70 mM boron.

According to plasmid DNA analysis, strain C161abᵀ had no plasmid content. Besides these findings, strain C161abᵀ was found to be sensitive to almost all the antibiotics tested: vancomycin (30 µg), kanamycin (30 µg), bacitracin (10 U),...
Table 1. Phenotypic characteristics of strain C161ab<sup>T</sup> and phylogenetically related species

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<td>DNA G+C content (% mol)</td>
<td>37.8</td>
<td>41.7</td>
<td>43.9</td>
<td>43.9</td>
<td>40.2</td>
<td>43.1</td>
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<tr>
<td>Major fatty acids</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
<td>iso-C15:0, iso-C17:1ω7c, iso-C17:0, C16:0</td>
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</tbody>
</table>

*Data from Derekova et al. (2007); †; Yamamoto et al. (2004); ‡; Correvois et al. (2012); ‡; Shafer et al. (2004); ‡; Pikuta et al. (2000); ‡; Dulger et al. (2004); ‡; Gul-Guven et al. (2008); ‡; Poli et al. (2006); ‡; Sung et al. (2002).

List of fatty acids detected in strain C161abT.}

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chloramphenicol (30 μg), rifampicin (30 μg), tetracycline (30 μg), penicillin G (10 U), neomycin (30 μg) and azithromycin (15 μg). But it was resistant to novobiocin (30 μg). Other differentiating characteristics are listed in Table 1.

The 16S rRNA gene sequence determined for strain C161abT was 1446 nt long with a DNA G+C content of 56.3 mol%. In phylogenetic analyses of the 16S rRNA gene sequences, both maximum-likelihood and neighbour-joining phylogenies, which were applied according to the Jukes–Cantor and Kimura two-parameter models, respectively, placed strain C161abT into a separate species within the genus *Anoxybacillus* with maximum support.

Since both phylogenetic analyses were consistent with each other, only the neighbour-joining tree, which was reconstructed according to the Jukes–Cantor model, is presented in Fig. 2. Strain C161abT displayed 16S rRNA gene sequence similarities of between 94.6 and 96.8% to all the type strains of the known species of the genus *Anoxybacillus*. Strain C161abT displayed the greatest sequence similarity to *A. rupiensis* DSM 17127T (96.8%), *A. voynovskienis* DSM 17075T (96.8%) and *A. caldiproteolyticus* DSM 15730T (96.6%). In addition, similarity values of 96.1% to *A. tepidamans* DSM 16325T, 96.0% to *A. flavithermus* DSM 2641T and *A. ayderensis* NCIMB 13972T, 95.9% to *A. kamchatkensis* DSM 14988T, *A. amylyticus* DSM 15939T and *A. mongoliensis* DSM 19169T, and 95.8% to *A. salvavilienis* DSM 22626T and *A. contaminans* DSM 15866T were recorded. Much lower similarity was found with other species of the genus *Anoxybacillus* with validly published names. Moreover, strain C161abT showed 95.6, 95.3 and 94.8% sequence similarity to *G. galactosidasius* DSM 18751T, *Geobacillus toebii* DSM 14590T and *Geobacillus thermoglucosidasius* DSM 2542T, respectively, among species from the genus *Geobacillus*.

Despite 16S rRNA gene sequence similarity of 96.8%, DNA–DNA hybridization analysis revealed low relatedness of strain C161abT to *A. rupiensis* (21.2% relatedness) and *A. voynovskienis* (16.5%), as expected. These genomic DNA relatedness values are far below the threshold value of 70% recommended for the separation of novel bacterial species (Stackebrandt et al., 2002). Therefore, strain C161abT is considered to represent a separate species in the genus *Anoxybacillus*.

Among the members of the genus *Anoxybacillus*, the highest genomic DNA G+C content is found in *A. gonensis* (57 mol%) and *A. thermarum* (53.5 mol%), (Belduz et al., 2003; Poli et al., 2009). *A. tengchongensis* (41.1 mol%), *A. flavithermus* (41.6 mol%) and *A. vitaminiphilus* (39.2 mol%) are the species with the lowest of G+C contents (Pikuta et al., 2000; Zhang et al., 2010, 2013). Therefore, a DNA G+C content of 37.8 mol% for strain C161abT was the lowest among all the species of the genus *Anoxybacillus* with validly published names (Table 1).

According to respiratory quinone analysis, strain C16abT contained 96% MK-7 as the predominant menaquinone
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Anoxybacillus. Furthermore, the cell membranes of strain C161abT and A. rupiensis contain the highest amount of total iso-branched fatty acids (>80%) when compared with the other species of the genus Anoxybacillus, but strain C161abT differs from A. rupiensis by means of its facultatively anaerobic growth. In addition, the cluster analysis of strain C161abT, based on rep-PCR and ITS-PCR genomic fingerprinting, revealed unique banding patterns different from those of its closest relatives. Therefore, the low 16S rRNA gene sequence similarities (<96.8%) and DNA–DNA relatedness between strain C161abT and closely related species as well as differences in some phenotypic characteristics, chemotaxonomic and fingerprinting analyses described above allowed us to differentiate strain C161abT from its closest relatives. On the basis of this evidence, the isolate was designated as a representative of a novel species, for which the name Anoxybacillus calidus sp. nov. is proposed.

**Description of Anoxybacillus calidus sp. nov.**

*Anoxybacillus* (ca’li.dus. L. masc. adj. calidus hot, referring to hot habitats).

Cells are Gram-stain-positive, motile, spore-forming, straight rods, 0.5–0.7 μm wide and 4–13 μm long. The cells occur singly. Colonies are in 1–3 mm in diameter, cream, opaque, circular, non-mucoid and have a smooth surface with regular margins and rounded edges. Subterminal to terminally located ellipsoidal endospores are formed in a non-swollen sporangia. Negative for catalase, but positive for oxidase. Grows well aerobically, but is facultatively anaerobic. Thermophilic; growth occurs at 35–70 °C (optimum 55 °C) and pH 6.5–9.0 (optimum 8.0–8.5). Grows with 0 to 2.5% (w/v) NaCl, optimally with 0.5%. Tolerates 5 to 70 mM boron. Acid is produced from maltose, D-fructose, D-glucose, lactose, sucrose, ribose and D-mannitol, but not from D-(+)-galactose, D-(+)-xylose, D-mannose, D-sorbitol, L-arabinose, raffinose or trehalose. Gas is produced from glucose. Protease and lipase activities are negative, but positive for amylase, methyl red test and starch hydrolysis. Gives negative reactions for utilization of urea, casein, citrate, tyrosine and gelatin, Voges–Proskauer reaction, indole, and H2S production or reduction of nitrate. The type strain is C161abT (=DSM 25520T =NCIMB 14851T). Isolated from a soil sample collected near Saraykoy-Buharkent thermal power plant located in Kizildere province of Denizli, Turkey. The G+C content of the genomic DNA of the type strain is 37.8 mol% (HPLC).

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**References**


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