Thermolongibacillus altinsuensis gen. nov., sp. nov. and Thermolongibacillus kozakliensis sp. nov., aerobic, thermophilic, long bacilli isolated from hot springs

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Two novel endospore-forming, aerobic bacilli, strains E173aT and E265T, were isolated from soil and sediment samples from Kozakli and Altinsu hot springs, Nevsehir (Turkey). Their young cells in the exponential phase of growth were motile, Gram-stain-positive, straight rods, 0.6–1.1 by 0.6–1.2 by 9.0–35.0 μm in size, but they became strikingly long, approximately 0.6–1.2 by 9.0–35.0 μm, after the stationary phase of growth. Cells varied in tests for oxidase, and had a weakly positive reaction for catalase. Both strains could grow between 40 and 70 °C, with optimal growth at 60 °C (E173aT) and 55 °C (E265T). Growth occurred within the range pH 5.0–11.0 with optimal growth at pH 9.0 (E173aT) and pH 8.5 (E265T). Strain E173aT grew within a salinity range from 0 to 1.5 % (w/v) NaCl with optimal growth at 0.5 %, while strain E265T grew within the range 0–5.0 % (w/v), with an optimum at 3.0 %. The new isolates differed from each other in some phenotypic and chemotaxonomic characters as well as repetitive extragenic palindromic element PCR (rep-PCR) fingerprints. 16S rRNA gene sequence similarities suggested distant relationships with other members of the family Bacillaceae (<95.8 %), although the two strains showed 97.5 % sequence similarity between them, and had 55 % relatedness by DNA–DNA hybridization. The DNA G+C contents were 44.8 (E173aT) and 43.5 mol% (E265T). Moreover, the chemotaxonomic data of E173aT and E265T presence of low amounts of meso-diaminopimelic acid, A1γ to A1γ′ cross-linkage types in peptidoglycan, fatty acids including iso-C15 : 0 (≥60 %), iso-C17 : 0 and C16 : 0 supported the consideration of these isolates as members of a novel genus. Based upon phenotypic, phylogenetic and chemotaxonomic characteristics, it is proposed that new isolates represent a novel genus, Thermolongibacillus gen. nov., with two novel species: Thermolongibacillus altinsuensis sp. nov. (type strain E265T = DSM 24979T = NCIMB 14850T) and Thermolongibacillus kozakliensis sp. nov. (type strain E173aT = DSM 24978T = NCIMB 14849T).

Over the last decade, examination of the diversity in 16S rRNA gene sequences of Gram-positive or variable-staining, endospore-forming, aerobic or facultatively anaerobic, rod-shaped bacteria revealed that they were phylogenetically very heterogeneous and that these bacilli have undergone major taxonomic rearrangements (Logan et al., 2009). With the accumulation of further sequence data, the genus Bacillus has been divided into more manageable and better-defined groups. Among the newly established or reclassified genera, the thermophilic bacilli fall into the genus Bacillus genetic groups 1 and 5 according to their 16S rRNA gene sequences (Ash et al., 1991; Rainey et al., 1994). In 2001, the thermophilic bacteria belonging to Bacillus genetic group 5 were reclassified as members of the genus Geobacillus (Nazina et al., 2001). At the time of writing, the thermophilic endospore-formers with growth optima within the temperature range 45–70 °C have been placed into the genera Bacillus (Cohn, 1872; Fritzte, 2004), Sulfolobacillus (Golovacheva & Karavaiko, 2005).

Abbreviations: DPG, diphasatidylglycerol; ITS, intergenic transcribed spacer; meso-Dpm, meso-diaminopimelic; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; rep-PCR, repetitive extragenic palindromic element PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains E265T and E173aT are FJ429590 and FJ430056, respectively.

Two supplementary figures and two supplementary tables are available with the online version of this paper.
1978), Alicyclobacillus (Wisotzkey et al., 1992), Aneurinibac- 
cillus, Brevibacillus (Shida et al., 1996), Anoxybacillus (Pikuta  
et al., 2000), Thermobacillus (Touzel et al., 2000), Geobacillus  
(Nazina et al., 2001), Ureibacillus (Fortina et al., 2001),  
Cerasibacillus (Nakamura et al., 2004), Caldalkalibacillus (Xue  
et al., 2006; Zhao et al., 2006) Aeribacillus (Michana-Galbis  
et al., 2010), and Caldibacillus (Coorevits et al., 2012).

During our previous polyphasic taxonomic study, we  
isolated more than 500 thermophilic bacilli from different  
gerothermal regions of Turkey (Coleri et al., 2009). Of those  
desorbosphere-forming bacteria, 115 of the bacilli were  
subjected to 16S rRNA gene sequencing and their phylo-
genetic analyses revealed the presence of two novel thermo-
philic, desorbosphere-forming, aerobic, long, rod-shaped  
bacteria: strains E265T and E173aT, isolated from soil and  
sediment samples collected from two different hot springs  
of Altinsu and Kozakli provinces in Nevsehir. Although  
these new isolates showed distant phylogenetic relation-
ships with some of the members of the genera Geobacillus  
and Anoxybacillus, they did not fit into any of the  
thermophilic genera previously described as belonging to  
the family Bacillaceae as they displayed very low (<95.8 %)  
16S rRNA gene sequence similarities (Cihan et al., 2011,  
2012). Therefore, they were taken into consideration for  
the novel genus, Thermolongibacillus gen. nov.,  
with two species, Thermolongibacillus altinsuensis sp. nov.  
and Thermolongibacillus kozakliensis sp. nov.

Strain E173aT was isolated from a soil sample collected  
beside the Kozakli Municipality thermal hot spring in  
Kozakli province of Nevsehir, Turkey, whereas strain E265T  
isolated from a sediment sample collected aseptically from  
Altinsu hot spring in Kozakli (34° 43' E 38° 38' N). Both  
of these hot springs were located in the Cappadocia  
area belonging to the Middle Anatolian Region of Turkey  
and the water temperature and pH of these thermal hot  
springs were measured to be 96 to 98 °C and pH 6.8–7.0  
to 7.5. The samples (0.1 g) were incubated at 60 °C with  
250 r.p.m. shaking for 24 h in 5 ml of Geobacillus  
thermobucidosans medium (MI broth) containing 1%  
soluble starch (pH 7.0) to obtain the enrichment culture  
(Suzuki et al., 1976). When the turbid enrichments were  
streaked on plates of MI containing 3% agar and incubated  
aerobically at 60 °C for 24 h, strains E173aT and E265T  
were the only dominant micro-organisms that we isolated.  
Cultures were purified from the same medium by subculturing four times and the purity was also confirmed  
microscopically. Although no other colony morphologies  
were observed, six to eight colonies of E173aT and E265T  
were picked and isolated, including the original strains. In  
order to avoid repeated examination, all the isolated clones  
were subjected to 16S rRNA gene sequencing, repetitive  
extragenic palindromic element PCR (rep-PCR) and  
intergenic transcribed spacer (ITS)-PCR analyses. In  
addition to similar colony morphologies, the 16S rRNA  
gen gene sequences and the fingerprinting profiles of the clones  
were the same as the original E173aT and E265T strains.  
Therefore, this paper describes the taxonomy of two novel  
species based upon single isolates. For long-term mainte-
nance, turbid cultures in MI broth were supplemented with  
20 % (w/v) glycerol and stored at −80 °C.

The temperature range for growth was determined by  
icubating the strains in nutrient broth (Merck 1.05443) at  
temperatures from 30 to 80 °C. The pH dependence was  
tested in nutrient broth which was adjusted with either  
NaOH or HCl to various pH values from 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 salinities was  
determined spectrophotometrically by measuring the OD  
at 600 nm. Cytology and colony morphology observations  
were determined aerobically under optimal growth condi-
tions depending on the isolate. Colony characteristics were  
determined in 18- to 72-hour-old cultures grown on MI  
and nutrient agar plates containing 3% agar. The  
formation of spores was tested by using cultures grown both  
in broth and on solid media of nutrient and MI media  
supplemented with 5 mg l⁻¹ MnSO₄, 4H₂O for 18–72 h and  
observed under a phase-contrast microscope (Olympus BX51)  
as in the case of motility tests. The morphological cell cycle was  
also photographed with a phase-contrast microscope by using  
cells grown on MI plates at 6, 18, 24, 72 h and 7 days. Gram  
staining was determined according to the methods of Claus  
& Berkeley (1986).

Physiological characterization tests included: anaerobic  
growth, oxidase and catalase activity, casein, citrate, starch,  
tyrosine, gelatin and urea utilization, reduction of nitrate  
to nitrite, N₂ gas production from nitrate, acid production  
from sugars, gas production from glucose, methyl red test,  
Voges–Proskauer test (pH 6.9), and indole and H₂S  
production. These tests were carried out by the methods  
of Claus & Berkeley (1986) and observed after 1, 2 and  
7 days of incubation. The organic substrate utilization tests  
were carried out by using a basal medium (Adkins et al.,  
1992) supplemented with separately sterilized aliphatic  
hydrocarbons (n-alkanes of C₅–C₁₀) and cyclic hydro-

carbons (cyclohexan) at 5 ml l⁻¹, aromatic hydrocarbons  
(naphthalene, naphthylamine) at 2.5 g l⁻¹ or 5 ml l⁻¹  
(benzene, toluene), organic acids (lactate, citrate, acetate,  
pyruvate, succinate, benzoate, butyrate, carbonate, trypt-
tone, peptone, yeast extract, phenol) at 2.5 g l⁻¹ and  
alcohols (ethanol, methanol, butanol, propanol, iso-
butanol, glycerol) at 10 mM. A disc diffusion test was  
performed by using nutrient agar plates for testing the  
antimicrobial susceptibility to vancomycin (30 μg), kan-
mycin (30 μg), novobiocin (30 μg), bacitracin (10 U),  
chloramphenicol (30 μg), rifampicin (30 μg), tetracycline  
(30 μg), penicillin G (10 U), neomycin (30 μg) and  
azithromycin (15 μg). Plasmid DNA isolation was carried  
out by the procedure of Anderson & McKay (1983). 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. For \(\alpha\)-glucosidase and \(\alpha\)-amylase assays, strains were incubated in 1% starch-containing MI medium for 24 h and the enzyme activities were measured by using spectrophotometric paranitrophenol \(\alpha\)-D-glucoside and dinitrosalicylic 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 paranitrophenol 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).

Strains were incubated in TSBA40 medium at 55 °C and then fatty acids 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). Fatty acids were further determined using GC, whereas quinones were analysed by HPLC. Polar lipids were extracted by a modified method of Bligh & Dyer (1959). Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.) and separated by two dimensional silica gel TLC (Rhuland et al., 1955; Tindall et al., 2007). In meso-diaminopimelic (meso-Dpm) acid content analysis, the whole cells of the E173a\(^T\) and E265\(^T\) were hydrolysed with 4 M HCl at 100 °C for 16 h and subjected to TLC on cellulose plates using the solvent system of Rhuland et al. (1955). All the fatty acid, quinone and cell wall composition analyses were determined at the DSMZ Identification Service, Braunschweig, Germany. The fatty acids, quinones and polar lipid tests were performed using cells grown on TSA.

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 Cashon et al. (1977). In determining G+C base composition of the genomic DNA, the DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed using HPLC. The G+C content of the genomic DNA was measured 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 55–60 °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).

For the genomic fingerprinting analyses of the repetitive extragenic palindromic (rep) elements, PCR was performed with the (GTG)\(5\) and BOXA1R primers using the conditions that were 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 ITS between 16S and 23S rRNA genes and PCR conditions were adjusted according to Daffonchio et al. (2003). The PCR products were analysed by electrophoresis in 1.5% agarose gel by using 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 DNA bands, their sizes and also on 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.

Colonies of strain E173a\(^T\) were 1–3 mm in diameter, non-mucoid, cream in colour, opaque, circular and convex, having regular margins with entire edges and a smooth surface when cultured on MI plates at the optimum growth temperature for 18–24 h. In the case of strain E265\(^T\), colony morphology differed depending on the culture age. The majority of the E265\(^T\) colonies (>60%) were circular, cream in colour, 2–3 mm in diameter and convex with entire edges and smooth surface after the first cultivation following long-term maintenance of cells such as storage at +4 °C for more than six months, lyophilization or freezing in glycerol-containing medium at −80 °C. Following two to three subcultures on MI plates at the optimal temperature for 18–24 h, these round colonies turned ellipsoidal in shape, similar to the rest of the colonies, became wider (2–6 mm width and 4–10 mm length), non-mucoid, light yellow in colour and flat with undulate edges, and had a rough surface and opaque centres with translucent edges. These active ellipsoidal colonies then became dominant on MI plates and formed the final colony morphology of strain E265\(^T\). When the clones taken from these different colony morphologies were subjected to microscopic examination, 16S rRNA gene sequencing and
The morphological cell cycle was observed and also photographed in a phase-contrast microscope by using cells grown on MI plates for 6, 18, 24 and 72 h and 7 days (Fig. S1, available in IJSEM Online). Microscopy of strains E173aT and E265T revealed that their cells were Gram-positive, motile, spore-forming, rods generally observed as singular, pairs or long chains of cells. Endospores were located terminally without swollen sporangium (Fig. S1a). Spore formation became detectable after 18 to 24 h of incubation on MI plates as shown in Fig. S1(b–e) and all the cells were sporulated after 72 h of incubation. The free spores were measured as 1.5 to 2 μm in length for both of the strains (Fig. S1f). Vegetative cells of strain E173aT had ellipsoidal to oval endospores in non-swollen sporangia, whereas, only ellipsoidal endospores occurred in non-swollen sporangia in the case of strain E265T. Young and straight cells in the lag and exponential phases of growth (6 to 18 h) had sizes of 0.6–1.0 × 3.0–8.0 μm and 0.7–1.1 × 3.5–8.0 μm in width and length for strains E173aT and E265T, respectively. Between stationary and death phases (20 to 50 h of incubation), the vegetative cells that were still not sporulated became strikingly longer, approximately 0.6–1.1 × 9.0–32.0 μm in width and length for strain E173aT cells and 0.8–1.2 × 9.0–35.0 μm for strain E265T (Fig. S1e). Nevertheless, when the cells were cultured on both nutrient and MI broths instead of plates, nearly half of the vegetative cells developed spores within 72 h, whereas the unseparated cells formed filaments. Long cell formation was also observed in liquid cultures of strains E173aT and E265T as in the case of solid cultures during 7 days of incubation. Therefore, as revealed by cell morphology studies, the occurrence of extremely long cells after the late exponential growth phase and also after the prolonged incubation was a salient characteristic for both strains E173aT and E265T among the other thermophilic endospore-forming bacilli.

The detailed physiological characteristics of strains E173aT and E265T are given in their species descriptions. In addition, some differentiating phenotypic characteristics of strains E173aT and E265T from each other and from phylogenetically related species belonging to the genera *Anoxybacillus* and *Geobacillus* are presented in Table 1. Both of the novel strains were found to be weakly positive for catalase activity. The oxidase activity of strain E173aT was found to be negative, but strain E265T gave a weakly positive reaction for oxidase. They both grew aerobically, but could not grow in anaerobic conditions. Both of the strains were thermophilic, growing within the range 40°C–70°C, with optimal growth at 60°C for strain E173aT and 55°C for strain E265T. They were moderate alkaliophiles with a pH range for growth between pH 5.0 and 11.0 with optimal pH at 9.0 and 8.5 for strains E173aT and E265T, respectively. There was also a difference in their NaCl requirements for growth. Although strain E173aT did not grow in NaCl concentrations of more than 1.5% (w/v), strain E265T grew with up to 5% NaCl (w/v). The following were utilized by both of the novel strains as carbon and energy sources: acetate, pyruvate, succinate, benzoate, tryptone, peptone, yeast extract and glycerol. They could not grow on lactate, citrate, carbonate, butanol, naphthalene or naphthylamine; nevertheless, poor growth was observed on substrates of butyrate, phenol and cyclohexan. Growth on n-alkanes (C₉–C₆₀) was not observed, but they slowly oxidized octane (C₈) in 7 days exclusively. Moreover, strains E173aT and E265T varied in the utilization of methanol, ethanol, propanol, isobutanol, benzene and toluene as described in their species descriptions. In addition, strain E173aT differed from strain E265T in other phenotypic characteristics such as growth in Sabouraud dextrose, urea utilization and acid production from lactose, glucose, galactose, sucrose, sorbitol, arabinose, raffinose and ribose.

Their plasmid DNA analyses revealed that E173aT and E265T strains contained plasmid bands of 14.5 and 15.5 kb, respectively. Both strains were sensitive to vancomycin, kanamycin, novobiocin, bacitracin, chloramphenicol, rifampicin, tetracycline, penicillin G, neomycin and azithromycin.

The dominant cellular fatty acids of strains E173aT and E265T were the iso-branched fatty acids iso-C₁₅:₀ and iso-C₁₇:₀ exceeding 70% of the total fatty acid profiles (Table 2). Although there were some slight differences, both of these strains contained a high amount of iso-C₁₅:₀ (>60%). Furthermore, the other representative thermophilic members of the family Bacillaceae, *Geobacillus toebii* DSM 14590T and *Anoxybacillus caldiproteolyticus* DSM 15730T, were also studied for their fatty acid contents (Table 2). Although the iso-branched fatty acids iso-C₁₅:₀ and iso-C₁₇:₀ were found to be their major membrane lipids, the amount of iso-C₁₅:₀ (32.19%, DSM 14590T; 42.03%, DSM 15730T) did not constitute more than half of their fatty acid contents as in the case of strains E173aT and E265T. The results of fatty acid analyses showed that species from the genera *Aeribacillus*, *Aeurinribacillus*, *Anoxybacillus*, *Caldalkalicibacillus*, *Caldibacillus* and *Geobacillus* contain iso-C₁₅:₀ as a major iso-branched fatty acid, but strains E173aT and E265T are unique as they their cell membranes contained a very high amount of this kind of fatty acid.

Strains E173aT and E265T shared the polar lipids diphostidyglycerol (DPG), phosphatidyglycerol (PG), phosphatidylethanolamine (PE) and two phospholipids (PL1, PL2) in their cell membranes, but strain E173aT differed from strain E265T by additional minor amounts of aminophospholipid (PN) and aminolipids (AL1, AL2). As can be seen from Table S2, in contrast to the members of the genera *Aeribacillus* and *Caldibacillus*, the species from the genera *Geobacillus* and *Anoxybacillus* contained DPG, PG and PE in common with strains E173aT and E265T, but they varied in their other polar lipid contents. Strains E173aT and E265T could be easily differentiated from the closely related strains *Geobacillus toebii* DSM 14590T and *G. thermoglucosidans* DSM 2542T by the...
**Table 1. Phenotypic characteristics of strains E173a\(^T\) and E265\(^T\) and phylogenetically related species from the genera *Geobacillus* and *Anoxybacillus***

Strains: 1, E173a\(^T\); 2, E265\(^T\); 3, Anoxybacillus caldiproteolyticus DSM 15730\(^T\); 4, *Anoxybacillus voinovskiensis* DSM 17075\(^T\); 5, *Anoxybacillus tepidamans* DSM 16325\(^T\); 6, *Geobacillus thermoglucosidans* DSM 2542\(^T\); 7, *Geobacillus caldoxylosilyticus* DSM 12041\(^T\); 8, *Geobacillus stearothermophilus* DSM 22\(^T\); 9, *Geobacillus toebii* DSM 14590\(^T\). All phenotypic characteristics were determined in this study. +, Positive; −, negative; w, weakly positive; v, variable; ND, not detected; T, terminal; St, subterminal; C, central; E, ellipsoidal; Ov, oval. All strains were found to be aerobic and positive for the Gram reaction, motility, and acid production from maltose and D-fructose. All strains were negative for citrate and tyrosine utilization, H\(_2\)S production in TSI, indole test and gas production from nitrate.

<table>
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<th>Characteristics</th>
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<td>(55)</td>
<td>(62)</td>
<td>(50–65)</td>
<td>(55)</td>
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<td>pH requirement (optimum)</td>
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<td>5.5–9.5 (7.0)</td>
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<td>5.0–10.0 (6.0)</td>
<td>6.0–8.0 (8.5)</td>
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absence of glycolipids (GL) and lipids (L), from *Geobacillus stearothermophilus* DSM 22^T^ by the lack of lipids (L), aminophosphoglycolipids (APGL) and phosphoaminolipids (PAL), and from *Anoxybacillus caldiproteolyticus* DSM 15730^T^ and *Anoxybacillus tepidamans* DSM 16325^T^ by the absence of lipids (L1–L4). (Table S1).

According to respiratory menaquinone analysis, the quinone content of strain E173a^T^ was completely composed of MK-7 (100 %). In the case of strain E265^T^, the respiratory menaquinones were found to be MK-7 (96 %), MK-6 (2 %), MK-5 (1 %) and MK-8 (1 %).

The whole-cell hydrolysaties of the novel strains contained meso-Dpm as diagnostic amino acid. The amount of meso-Dpm was found to be relatively low in the cell walls of strains E173a^T^ and E265^T^, as well as to other members of the family *Bacteroidaceae* including the thermophilic endospore-formers of the genera *Bacillus*, *Brevibacillus*, *Geobacillus*, *Sulfobacillus* and *Caldisporon*, whereas other species from the genera *Anoxybacillus* and *Geobacillus*, and some representative endospore-forming bacilli are shown in Fig. S2. Although strains E173a^T^ and E265^T^ were found to be most closely related to each other with 97.5 % similarity of 16S rRNA gene sequence. Furthermore, strain E173a^T^ showed distant sequence similarity values to *G. toebii* DSM 14590^T^ (94.4 %), *Geobacillus galactosidasius* DSM 18751^T^ (94.3 %), *Bacillus thermodenitrificans* DSM 9572^T^ (93.8 %) and *Anoxybacillus caldiproteolyticus* DSM 12041^T^ (93.7 %); *G. thermoglucosidas* DSM 2542^T^ (93.8 %) and *Anoxybacillus caldiproteolyticus* DSM 15730^T^ (93.3 %), whereas the similarity values for the 16S rRNA gene sequence of strain E265^T^ were found to be 95.7 %, 95.6 %, 95.6 %, 95.2 %, 95.1 %, 95.0 % and 94.9 %. The phylogenetic tree based on 16S rRNA gene sequences containing the most closely related species to the new isolates is presented in Fig. 1; the tree was reconstructed using the neighbour-joining method. The tree was composed of three major clusters containing the thermophilic members of *Bacillus* genetic group 5; species from the genera *Anoxybacillus* and *Geobacillus*, and a third cluster containing novel isolates E173a^T^ and E265^T^.

According to the 16S rRNA gene sequence similarity values, there were no closer relatives of the novel strains as their similarity to each other was 97.5 %. In addition, the ad hoc committee recommends a threshold value of 70 % DNA–DNA relatedness for the definition of novel bacterial species which show 97 % or greater 16S rRNA gene sequence similarity (Stackebrandt et al., 2002). Thus, when strains E173a^T^ and E265^T^ were subjected to DNA–DNA hybridization analysis according to the recommendations, only a 55 ± 4.7 % (mean ± SD) DNA reassociation value was found between these two strains. A 55 % DNA–DNA hybridization value between E173a^T^ and E265^T^ strains revealed that they could not belong to the same species. Furthermore, the G+C content of the genomic DNA of strain E173a^T^ was 44.8 mol%, whereas this value was found to be 43.5 mol% for strain E265^T^.

The BOX-, (GTG)3-, and ITS-PCR fingerprinting patterns, and their cumulative cluster analyses containing strains E173a^T^ and E265^T^ and some representative endospore-forming bacilli are shown in Fig. S2. Although strains E173a^T^ and E265^T^ could not be differentiated by means of their ITS fingerprinting profiles, all the reference type strains and also strains E173a^T^ and E265^T^ demonstrated unique banding patterns according to the rep-PCR fingerprints. In (GTG)_3_-PCR profiles, the discriminative DNA bands of 2641, 2034 and 1338 bp were found only in strain E173a^T^, whereas strain E265^T^ harboured a 1360 bp DNA band which did not exist in E173a^T^. Moreover, the BOX-PCR analyses revealed 648 and 490 bp unique DNA bands peculiar to strain E265^T^, while a 1670 bp band was only present in strain E173a^T^. On the basis of the cumulative cluster analysis including three of these
fingerprinting tests, strain E173a<sup>T</sup> displayed a similarity value of 95.5% with its close relative strain E265<sup>T</sup>. In addition, when the similarities obtained from the other representative type strains were used, the highest values found were 66.1% for strain E173a<sup>T</sup> and 68.9% for strain E265<sup>T</sup> with Anoxybacillus tepidamans DSM 16325<sup>T</sup>. As can be deduced from the fingerprinting analyses, the novel strains mostly resembled each other, although they showed differences and formed a different cluster among the other type strains compared. Thus, as the rep-PCR can be used to discriminate strains at the species and subspecies level (Versalovic et al., 1994), the distinctive rep-PCR bands allowed us to show the genetic diversity of strains E173a<sup>T</sup> and E265<sup>T</sup> not only from each other, but also from all the other species as they came together in order to form a distinct genetic cluster.

In conclusion, the most distinctive characteristics of these two strains were their extremely long and slim cell morphologies in cultures between the stationary and death growth phases as their cell lengths reached up to 32.0–35.0 μm, before the vegetative cells on plates were completely sporulated. Furthermore, they also showed differences to other genera including the thermophilic members of the family Bacillaceae in many aspects and some of these differentiating characteristics are summarized in Table S2. On the basis of phenotypic, phylogenetic, chemotaxonomic and genetic characteristics presented in this paper, a new genus with the name referring to thermostability and tendency to form long chains of cells, Thermolongibacillus gen. nov. within the family Bacillaceae is proposed; this new genus accommodates two novel species, Thermolongibacillus altissuensis E265<sup>T</sup> sp. nov. and Thermolongibacillus kozakliensis E173a<sup>T</sup> sp. nov.

**Description of Thermolongibacillus gen. nov.**

Thermolongibacillus (Ther.mo.longi.bacillus. Gr. adj. thermos hot; L. adj. longus long; L. dim. n. bacillus small rod; N.L. masc. n. Thermolongibacillus long thermophilic rod).

Cells are Gram-stain-positive, motile, spore-forming, straight rods, occurring singly, in pairs, or in long straight or slightly curved chains. Young cells are between 0.6–1.1 μm in width and 3.0–8.0 μm in length; the occurrence of slim and extremely long cells, approx. 0.6–1.2 by 9.0–35.0 μm, between stationary and death phases is a salient characteristic. Colony morphology is peculiar to species. Terminally located ellipsoidal to oval endospores are formed in non-swollen sporangia. Thermophilic. Grow within a temperature range from 40 to 70 °C. Moderately alkaliphilic with a broad pH range of 5.0–11.0. NaCl tolerance varies according to species. Grow aerobically, but not in anaerobic conditions. Weakly positive reaction for catalase activity, but oxidase activity is variable. Hydrolyse sugars. Variable for urea utilization and growth on Sabouraud dextrose as well as acid production from lactose, D-glucose, D- (+)-galactose, sucrose, D-sorbitol, D-arabinose, raffinose and

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<th>Fatty acid content (%)</th>
<th>E173a&lt;sup&gt;T&lt;/sup&gt;</th>
<th>E265&lt;sup&gt;T&lt;/sup&gt;</th>
<th>DSM 14590&lt;sup&gt;T&lt;/sup&gt;</th>
<th>DSM 15730&lt;sup&gt;T&lt;/sup&gt;</th>
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<td>0.14</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>99.99</td>
<td>100</td>
<td>99.97</td>
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The major fatty acids are iso-C_15 : 0, iso-C_17 : 0 and C_16 : 0, of amphenicol, rifampicin, tetracycline, penicillin G, neomycin to vancomycin, kanamycin, novobiocin, bacitracin, chlor-carbonate, butanol, naphthalene or naphthylamine. Sensitive phenol, octane and cyclohexan. No growth on lactate, citrate, carbon and energy sources, but poorly oxidize butyrate, benzoate, tryptone, peptone, yeast extract and glycerol as sole sources. The predominant menaquinone is MK-7.

The diagnostic amino acid in murein structure is iso-C_15 : 0 constituting more than 60 % of all the fatty acids. The predominant menaquinone is MK-7. The major fatty acids are iso-C_15 : 0, iso-C_17 : 0 and C_16 : 0, of which iso-C_15 : 0 constitutes more than 60 % of all the fatty acids. The diagnostic amino acid in murein structure is meso-Dpm in low amounts. Contain peptidoglycan types A1_c, A1_i and A1_e (A31) and A1_γ (A32.1) in cross-linkages. Cell membranes contain DPG, PG, PE and two phospholipids, aminophospholipids and two aminolipids. The intragenic 16S rRNA gene sequence similarity is 97.5 % and species can be differentiated by means of their rep-PCR fingerprints. The G+C contents are between 43.5 and 44.8 mol%. The type species is Thermolongibacillus altinsuensis.

Description of Thermolongibacillus altinsuensis sp. nov.

Thermolongibacillus altinsuensis [al.tin.su.en’sis. N.L. masc. adj. altinsuensis pertaining to the isolation habitat, Altinsu hot spring located in Kozakli province of Nevsehir in the Middle Anatolian Region of Turkey (Cappadocia area)].

In addition to the characteristics given above for the genus, cells are Gram-stain-positive, motile, straight, slim rods measuring 0.7–1.1 by 3.5–8.0 µm in young cultures. Cells occur singly. Rod cells form chains and are strikingly longer in size between stationary and death phases (0.8–1.2 by 9.0–35.0 µm). The free ellipsoidal to oval spores are 1.5 to 2 µm. Long-term incubated colonies are circular, cream in colour, 2–3 mm in diameter, and convex with entire edges and smooth surface, whereas actively growing colonies are ellipsoidal, light yellow, flat with undulate edges, non-mucoid, have a rough surface, and are 2–6 × 4–10 mm. Moderately thermophilic. Growth occurs at 40–70 °C (optimum 55 °C) and pH 5.0–11.0 (optimum 8.5). Grows with 0 to 5.0 % (w/v) NaCl, optimal growth with 3 %. Catalase and oxidase reactions are weakly positive. Positive for casein and urea utilization, and methyl red test, but negative for the entire phenotypic tests including starch, citrate, tyrosine and gelatin utilization, growth on Sabouraud dextrose, Voges–Proskauer test, and indole and H_2S production. Reduction of nitrate to nitrite is positive, but gas is not produced from nitrate. Acid is produced from maltose, D-fructose, lactose, D-(+)-galactose, D-(-)-xylose, D-mannose, D-sorbitol, L-arabinose, D-mannitol and raffinose, but not from D-glucose, sucrose, trehalose or ribose. Utilizes isobutanol, weakly oxidizes methanol and toluene, but cannot grow on ethanol, propanol or benzene as sole carbon and energy sources. Negative for amylase, x-glucosidase, protease and lipase activities. Contains a 15.5 kb plasmid band. The major menaquinone is MK-7 in addition to minor amounts of MK-6, MK-5 and MK-8.
The predominant fatty acids are iso-C$_{15:0}$, iso-C$_{17:0}$ and C$_{16:0}$. Cell membrane contains the polar lipids DPG, PG, PE and two phospholipids.

The type strain is E265$^T$ (= DSM 24979$^T$=NCIMB 14850$^T$), isolated from a sediment sample of Altinsu hot spring in Kozakli province of Nevsehir, Turkey (Cappadocia Area). The DNA G+C content of the type strain is 43.5 mol%.

**Description of Thermolongibacillus kozakliensis sp. nov.**

*Thermolongibacillus kozakliensis* [ko.zak.li.en’sis. N.L. masc. adj. kozakliensis pertaining to the isolation habitat, Kozakli Municipality hot spring located in Kozakli province of Nevsehir in the Middle Anatolian Region of Turkey (Cappadocia area)].

Cells are Gram-stain-positive, motile, straight, slim rods, which generally occur singly, approximately 0.6–1.0 by 3.0–8.0 pm in young cultures. Form long chains. Extremely longer in sizes (0.6–1.1 by 9.0–32.0 pm) between stationary and death phases. The free ellipsoidal spores are 1.5 to 2 pm in length. Colonies are in 1–3 mm in diameter, non-mucoid, cream in colour, opaque, circular and convex, having regular margins with entire edges and smooth surface. Thermophilic. Growth occurs between 40 °C and 70 °C (optimum 60 °C), at pH 5.0-11.0 (optimum pH 9.0) and at salinities from 0 to 1.5 % (w/v) (optimum 0.5 %, w/v). Catalase reaction is weakly positive. Negative for oxidase activity. Gives positive reactions for casein utilization, growth on Sabouraud dextrose and the methyl red test, but negative reactions for other phenotypic tests such as starch, citrate, tyrosine, dextrose and the methyl red test, but negative reactions for casein utilization, growth on Sabouraud dextrose and the methyl red test, but negative reactions for other phenotypic tests such as starch, citrate, tyrosine, dextrose and the methyl red test, but negative reactions for casein utilization, growth on Sabouraud dextrose and the methyl red test, but negative reactions for other phenotypic tests. Hydrolyses sugars. Acid is produced from maltose, D- (+)-xylose, D-mannose, ribose and D-galactose, but not from D-(+)-galactose, D-sorbitol, trehalose or raffinose. Can produce acid from D-glucose, D-mannitol, but not from D-(+)-glucose, D-glucose, but gas production is negative. Can weakly use L-arabinose and lactose. Utilizes ethanol; growth is weak on propanol, isobutanol and benzene; cannot oxidize methanol or toluene as sole carbon and energy sources. Negative for amylase, $\alpha$-glucosidase, protease and lipase activities. Has a plasmid band of 14.5 kb. The menaquinone content is MK-7. Fatty acid profile is largely composed of iso-C$_{15:0}$, iso-C$_{17:0}$ and C$_{16:0}$. Predominant polar lipids are DPG, PG, PE, two phospholipids, aminophospholipids and two aminolipids. Other characteristics are as given above in the genus description.

The type strain is E173$^T$ (= DSM 24978$^T$=NCIMB 14849$^T$), isolated from a soil sample of Kozakli Municipality Thermal hot spring located in Kozakli province of Nevsehir, Turkey (Cappadocia Area). The DNA G+C content of the type strain is 43.8 mol%.

**Acknowledgements**

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**References**


