Alicyclobacillus consociatus sp. nov., isolated from a human clinical specimen

Stefanie P. Glaeser,1 Enevold Falsen,2 Karin Martin3 and Peter Kämpfer1

1Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany
2Culture Collection University Göteborg, Dept of Clinical Bacteriology, S-41346 Göteborg, Sweden
3Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie e.V., Hans-Knöll-Institut, D-07745 Jena, Germany

A Gram-stain-positive, aerobic organism, isolated from a blood sample from a 51-year-old woman, was studied for its taxonomic position. Based on 16S rRNA gene sequence similarity comparisons, strain CCUG 53762T was grouped into the genus Alicyclobacillus, most closely related to the type strain of Alicyclobacillus pohliae (94.7%). The 16S rRNA gene sequence similarity to other species of the genus Alicyclobacillus was ≤91% and similarity to species of the genus Tumebacillus was 91.3–93%. The occurrence of menaquinone MK-7 as the major respiratory quinone, meso-diaminopimelic acid as the diagnostic diamino acid of the cell wall and the fatty acid profile supported the allocation of the strain to the genus Alicyclobacillus. Major fatty acids were iso- and anteiso-branched fatty acids. The polar lipid profile consisted of diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three unknown phospholipids. The absence of the iso-branched fatty acids iso-C16 : 0 and iso-C17 : 0 allowed differentiation of strain CCUG 53762T from A. pohliae CIP 109385T. In addition, the results of physiological and biochemical tests also allowed phenotypic differentiation of strain CCUG 53762T from this most closely related species. The G+C content of the DNA was 47 mol%. Strain CCUG 53762T therefore represents a novel species of the genus Alicyclobacillus, for which we propose the name Alicyclobacillus consociatus sp. nov., with CCUG 53762T (=CCM 8439T) as the type strain.

The genus Alicyclobacillus, proposed by Wisotzkey et al. (1992), now accommodates more than 20 species. Members of this genus are Gram-stain-positive, spore-forming, thermophilic and heterotrophic organisms, which have been found in various extreme habitats like hot springs (Alicyclobacillus vulcanalis, Simbahan et al., 2004), geothermal soil (Alicyclobacillus pohliae, Imperio et al., 2008) and acidic environments (Alicyclobacillus acidocaldarius; Wisotzkey et al., 1992; Jiang et al., 2008), but can also live in soil and on plants such as fruits and crops (Goto et al., 2002, 2007). The genus Alicyclobacillus is remarkably heterogeneous. Initially described as a genus characterized chemotaxonomically by the presence of large amounts of alicyclic fatty acids (Wisotzkey et al. 1992), many species have since been described, among them Alicyclobacillus aeris, Alicyclobacillus ferroxidans, Alicyclobacillus pomorum, Alicyclobacillus macrosorangiids, Alicyclobacillus contaminans and Alicyclobacillus pohliae (Goto et al., 2007; Guo et al., 2009; Imperio et al., 2008; Jiang et al., 2008), in which these characteristic fatty acids have not been reported.

In 2007, an endospore-forming organism was isolated from a blood sample from a 51-year-old woman on blood agar at 37 °C. Unfortunately, no other information of the clinical relevance of the strain is available. The strain was isolated from a blood culture, but it is not clear, and no proof has been documented, whether the strain really was the causal agent of an infection.

The organism was subcultivated on R2A agar (Oxoid) at 30 °C for 24 h. Gram-staining was performed according to the Hucker method as described by Gerhardt et al. (1994). Cell morphology was observed under a light microscope (Zeiss) at a magnification of ×1000 using cells that had been grown for 3 days at 25 °C on R2A agar. Cells of strain CCUG 53762T were rod-shaped (0.8–1.0 μm wide and 2–5 μm long, sometimes forming filamentous cells) and (rarely) formed terminal spherical endospores, grew strictly aerobically and formed beige, circular and convex colonies on the surface of R2A agar. Cells were non-motile and
stained Gram-positive. No cellular inclusions were observed.

For 16S rRNA gene sequencing and determination of the G+C content of the DNA, DNA was isolated from biomass of strain CCUG 53762^T with a commercial DNA extraction kit (GenElute Plant Genomic DNA kit; Sigma). The G+C content was determined by using a fluorometric thermal denaturation method as described by Gonzalez & Saiz-Jimenez (2002). Analysis was performed in a total volume of 20 µl including 5 µg genomic DNA, 0.1 × SSC buffer (0.03 M NaCl, 0.03 M sodium citrate), 10% (v/v) deionized formamide and 0.25 × SybrGreen I (Molecular Probes). Thermal denaturation was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) starting with 15 min at 25 °C, followed by a ramp from 65 to 98 °C with an increase of 0.1 °C per cycle. Each temperature was held for 5 s to allow a fluorescence measurement (SYBR channel). Analysis was performed at least in duplicate. Five references strains were used to generate a standard curve of DNA G+C content versus melting temperature \( T_m \). A linear regression analysis was used to calculate the DNA G+C content. The DNA G+C content of strain CCUG 53762^T was 47 mol%. This is relatively low in comparison with DNA G+C contents determined for species of the genus *Alicyclobacillus* (53.9–62.5 mol%), including the type species, at 61.9 mol% (Goto et al., 2007). The DNA G+C content of strain CCUG 53762^T is clearly different from that of the most closely related species, *A. pohliae* (55.1 mol%; Imperio et al., 2008).

The 16S rRNA gene was amplified by PCR and sequenced using universal primers 27f and 1492r (Lane, 1991) resulting in a continuous stretch of 1464 unambiguous nucleotides [positions 23–1490, according to *Escherichia coli* numbering; Brosius et al. (1978)]. Phylogenetic analyses were performed in ARB release 5.2 (Ludwig et al., 2004) using the All-Species Living Tree Project (LTP; Yarza et al., 2008) database release LTPs106 (August 2011). Sequences not included in the LTP database were aligned with SINA (version 1.2.9) according to the SILVA seed alignment (http://www.arb-silva.de; Pruesse et al. 2007) or downloaded from the SILVA homepage and implemented in the database. The alignment was adjusted manually based on secondary structure information. Sequence similarities were calculated without the use of an evolutionary substitution model. Phylogenetic trees were reconstructed with the maximum-likelihood method using RAxML version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, the neighbour-joining method with the Jukes–Cantor correction (Jukes & Cantor, 1969) and the maximum-parsimony method using DNAPARS version 3.6 (Felsenstein, 2005). All phylogenetic trees were calculated with 100 resamplings (bootstrap analysis; Felsenstein, 1985) and based on 16S rRNA gene sequences between *E. coli* positions 56 and 1458.

Sequence similarity calculations indicated that strain CCUG 53762^T was most closely related to the type strain of *A. pohliae* (94.7%) followed by the type strains of *Tumebacillus ginsengisoli* (93.4%) and *Tumebacillus permanentifrorigis* (91.3%). Sequence similarities to type strains of other *Alicyclobacillus* species ranged from 87.4 to 90.7%, with the highest similarity to *A. pomerum* 3A^T and 88.9% similarity to the type strain of the type species, *A. acidocaldarius*. Phylogenetic calculations showed that, independent of the treeing method used, strain CCUG 53762^T formed a distinct cluster with *A. pohliae* MP4^T (>70% bootstrap support; Fig. 1). In maximum-likelihood (Fig. 1) and maximum-parsimony trees, the cluster of the two strains was located between the genera *Alicyclobacillus* and *Tumebacillus*. In the neighbour-joining analysis, the two strains clustered closer to species of the genus *Tumebacillus*; however, this clustering was not supported by a high bootstrap value.

Based on the phylogenetic analysis, strain CCUG 53762^T and its closest relatives *A. pohliae* CIP 109385^T, *T. ginsengisoli* DSM 18389^T (Steven et al., 2008), *T. permanentifrorigis* DSM 18773^T (Baek et al., 2011) and the type strain of the type species of the genus, *A. acidocaldarius* DSM 446^T, were cultivated for further chemotaxonomic analyses.

For biomass production, strain CCUG 53762^T as well as *T. ginsengisoli* DSM 18389^T and *T. permanentifrorigis* DSM 18773^T were cultivated on R2A at 28 °C for 2 days, *A. pohliae* CIP 109385^T on R2A at 55 °C for 24 h and *A. acidocaldarius* DSM 446^T at pH 3–4 at 60 °C on medium 402 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium402.pdf) for 2 days. Determination of the isomers of dianimopimelic acid in whole-organism hydrolysates was performed as described by Hasegawa et al. (1983). Menaquinones were extracted and analysed as described by Collins et al. (1979) and Groth et al. (1996). Polar lipids extracted by the method of Minnikin et al. (1979) were identified by two-dimensional TLC as described by Collins & Jones (1980).

Whole-organism hydrolysates of the investigated strains contained meso-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan, with the major menaquinone MK-7 (95–97%) and traces of MK-6 (2–4%). All investigated strains contained the diagnostic polar lipids diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. In contrast to the two *Tumebacillus* strains and *A. pohliae* CIP 109385^T, strain CCUG 53762^T and *A. acidocaldarius* DSM 446^T contained only traces of phosphatidylglycerol. Phosphatidylserine was found in *T. ginsengisoli* DSM 18389^T and *T. permanentifrorigis* DSM 18773^T but not in *A. acidocaldarius* DSM 446^T, *A. pohliae* CIP 109385^T or strain CCUG 53672^T. The acidophilic strain *A. acidocaldarius* DSM 446^T contained a series of characteristic glycolipids not found in the strains of species of the genus *Tumebacillus, A. pohliae* CIP 109385^T or strain CCUG 53672^T; however, the phospholipid pattern of strain CCUG 53672^T was very similar to that of its closest phylogenetic neighbour *A. pohliae* CIP
Alicyclobacillus consociatus sp. nov.

**Fig. 1.** Maximum-likelihood tree showing the phylogenetic position of strain CCUG 53762<sup>T</sup> relative to type strains of all species of the genera *Alicyclobacillus* and *Tumebacillus*. The tree was reconstructed in ARB using RAxML (GTR-GAMMA, rapid bootstrap analysis, 100 bootstraps) and based on 16S rRNA gene sequences between positions 56 and 1458 (*E. coli* numbering; Brosius et al., 1978). GenBank accession numbers are given in parentheses. Numbers at branch nodes refer to bootstrap values >70% (100 replicates). Asterisks indicate nodes that were also found with high bootstrap support in neighbour-joining and maximum-parsimony analyses. *Bacillus subtilis* DSM 10<sup>T</sup> was used as an outgroup. Bar, 0.10 substitutions per nucleotide site.

Strain CCUG 53762<sup>T</sup> and could be distinguished from it only by the amount of phosphatidylglycerol (Fig. S1, available in IJSEM Online).

Fatty acid analysis of cells grown in R2A at 28 ºC or in M1209 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1209.pdf) was done as described by Kämpfer & Kroppenstedt (1996). We had to use different cultivation conditions (temperatures) because there was no single growth condition that allowed growth of all of the strains under comparison; however, biomass of all strains was harvested after 48 h of growth.

The fatty acids of strain CCUG 53762<sup>T</sup> comprised mainly iso- and anteiso-branched fatty acids and the profile was similar to those of the most closely related species of the genus *Alicyclobacillus*, but it was obvious that strain CCUG 53762<sup>T</sup> produced larger amounts of iso-branched fatty acids, in particular iso-C<sub>15:0</sub> and iso-C<sub>16:0</sub>. The detailed fatty acid profile obtained from cells grown on R2A medium after 72 h of incubation at 28 ºC is shown in Table 1. We did not detect ω-cyclic fatty acids. This is consistent with findings in other species such as *A. aeris*, *A. ferrooxydans*, *A. pomorum*, *A. macrosporangiidus*, and *A. pohliae* (Guo et al., 2009).

The results of the physiological characterization, performed using methods described previously (Kämpfer, 1990; Kämpfer et al., 1991), are given in Table 2 and in the species description. Strain CCUG 53762<sup>T</sup> was not able to produce acids from any sugars or sugar-related compounds tested, but was able to utilize several of them as sole sources of carbon. This distinct physiological biochemical profile allowed differentiation of the strain from the type strain of *A. pohliae*.

Based on the low 16S rRNA gene sequence similarities (<95 %) to all other species of the genus with validly published names, DNA–DNA hybridizations were not performed. From the results of the phylogenetic and chemotaxonomic analyses, it is obvious that strain CCUG 53762<sup>T</sup> represents a novel species of the genus *Alicyclobacillus*. For this species, we propose the name *Alicyclobacillus consociatus* sp. nov. The minimal standards provided by Logan et al. (2009) were considered.

**Description of Alicyclobacillus consociatus** sp. nov.

*Alicyclobacillus consociatus* (con.so.ci.a’tus. L. masc. adj. consociatus associated with, intended to mean that the type strain may have been associated with a human clinical case).

Cells are Gram-stain-positive, strictly aerobic rods (0.8–1.0 × 2.0–5.0 μm) and non-motile. Spherical endospores are (rarely) formed in a terminal position. Colonies grown on R2A agar are circular, convex and beige. Optimal temperature for growth is 30 ºC; growth occurs at 10–37 ºC. Cells are catalase- and oxidase-positive. Nitrates are not reduced. The cultivation conditions (temperatures) because there was no single growth condition that allowed growth of all of the strains under comparison; however, biomass of all strains was harvested after 48 h of growth.

http://ij.sgmjournals.org
Table 1. Cellular fatty acid contents after growth on (1,2) R2A agar or (3,4) M1209 medium of (1,3) strain CCUG 53762\textsuperscript{T} and (2,4) \textit{A. pohliae} CIP 109385\textsuperscript{T}

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>0.8</td>
<td>–</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>C15:0</td>
<td>–</td>
<td>2.4</td>
<td>–</td>
<td>1.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>3.9</td>
<td>2.7</td>
<td>2.4</td>
<td>2.9</td>
</tr>
<tr>
<td>iso-C14:0</td>
<td>7.5</td>
<td>3.0</td>
<td>12.8</td>
<td>–</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>12.9</td>
<td>39.4</td>
<td>24.7</td>
<td>43.1</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>–</td>
<td>14.0</td>
<td>15.1</td>
<td>4.6</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>7.5</td>
<td>19.6</td>
<td>4.8</td>
<td>32.9</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>20.4</td>
<td>10.3</td>
<td>11.5</td>
<td>8.5</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>2.7</td>
<td>6.2</td>
<td>–</td>
<td>4.4</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown ECL 14,263</td>
<td>–</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C16:1\textsuperscript{10}c</td>
<td>1.5</td>
<td>–</td>
<td>13.9</td>
<td>–</td>
</tr>
<tr>
<td>iso-C16:1\textsuperscript{H}</td>
<td>8.4</td>
<td>–</td>
<td>10.8</td>
<td>–</td>
</tr>
<tr>
<td>iso-C17:1\textsuperscript{9}c</td>
<td>0.9</td>
<td>–</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td>iso-C17:1\textsuperscript{10}c</td>
<td>–</td>
<td>–</td>
<td>2.3</td>
<td>–</td>
</tr>
<tr>
<td>iso-C18:1\textsuperscript{H}</td>
<td>1.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Summed features*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summed feature 1</td>
<td>–</td>
<td>–</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td>Summed feature 3</td>
<td>2.2</td>
<td>–</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td>Summed feature 4</td>
<td>9.3</td>
<td>–</td>
<td>10.5</td>
<td>–</td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 1 contains iso-C\textsubscript{15:1} \text{I} and/or C\textsubscript{15:0} 3-OH; summed feature 3 contains C\textsubscript{16:1}\textsuperscript{10}c and/or iso-C\textsubscript{15:0} 2-OH; summed feature 4 contains iso-C\textsubscript{16:1} \text{I} and/or anteiso-C\textsubscript{16:1} \text{B}.

Fatty acid analysis was performed with the standard MIDI procedures. Strain CCUG 53762\textsuperscript{T} was grown at 28 °C and \textit{A. pohliae} CIP 109385\textsuperscript{T} was grown at 55 °C. Data are expressed as percentages of total fatty acids. –, Not detected; ECL, equivalent chain length.

15–45 °C but not at 10 or 50 °C on R2A agar. Optimal pH for growth is 6.5; growth occurs at pH 5.5–10.5. Growth occurs in the presence of 1–2% NaCl but not at higher concentrations in medium M1209. Test for catalase is negative; tests for oxidase activity and H\textsubscript{2}S production are weakly positive. Tests for urease, degradation of gelatin and casein, arginine dihydrolase, lysine decarboxylase, 2-keto glutarate formation, benzoate, putrescine, acetate, propionate, cis- and trans-aconitate, adipate, azelate, citrate, itaconate, 2-oxoglutarate and mesaconate are not utilized as sole carbon sources. Major fatty acids are iso-C\textsubscript{15:0}, iso-C\textsubscript{16:0} and anteiso-C\textsubscript{15:0}. In addition, C\textsubscript{16:0} and iso-C\textsubscript{17:0} are detected. No \(\omega\)-cyclic fatty acids are detected. The diagnostic diamino acid of the peptidoglycan is \textit{meso}-diaminopimelic acid and the major menaquinone is MK-7. The major phospholipids are diphasphatidylglycerol, phosphatidylylycerol and phosphatidylethanolamine.

The type strain, CCUG 53762\textsuperscript{T} (=CCM 8439\textsuperscript{T}), was isolated from a blood sample from a 51-year-old woman. The DNA G+C content of the type strain is 47 mol%.

### Acknowledgements

We thank Gundula Will, Maria Sowinsky, Katja Grebing and Carmen Schulte for excellent technical assistance.

### References


