**Bacillus formosensis** sp. nov., isolated from pesticide wastewater

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A Gram-stain-positive, endospore-forming rod (designated strain CC-LY275ᵀ) was isolated from a pesticide wastewater sample. The isolate grew at a temperature 20–45 °C, at pH 7.0–8.0 and tolerated NaCl 6% (w/v). The most closely related strains in terms of 16S rRNA gene sequence similarity were *Bacillus horneckiae* (97.1%) and *Bacillus oceaniseudiminis* (96.8%), respectively. The G + C content of the genomic DNA was 37.9 mol%. Strain CC-LY275ᵀ was determined to possess iso-C₁₄:₀, iso-C₁₅:₀ and anteiso-C₁₅:₀ as predominant fatty acids. The major polar lipid profile consisted of diphasphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. Cell-wall peptidoglycan contained meso-diaminopimelic acid; menaquinone (MK-7) was the major respiratory quinone. According to the distinct phylogenetic, phenotypic and chemotaxonomic properties, the name *Bacillus formosensis* sp. nov. (type strain CC-LY275ᵀ=BCRC 80443ᵀ=JCM 18448ᵀ) is proposed.

*Bacillus subtilis* was the first species of the genus *Bacillus* and described by Ferdinand Cohn in 1872. The genus *Bacillus* belongs to the family *Bacillaceae* of the order *Bacillales*, most recognized species are represented by Gram-stain-positive, aerobic or facultatively anaerobic rods. Typical characteristics of members of the genus *Bacillus* are low DNA G + C content and containing menaquinone MK-7 as the major respiratory quinone. Due to their capacity to form endospores, they can survive long periods of high temperature (Cohn, 1872). Most species of the genus *Bacillus* prefer neutral pH and low salt concentrations, whereas some can also grow in environments with high alkalinity (Denizci et al., 2005) or high salinity (Pappa et al., 2010). Recently, different novel species such as *Bacillus oleivorans* (Azmatunnisa et al., 2015), *Bacillus crassostreae* (Chen et al., 2015), *Bacillus encimensis* (Dastager et al., 2015) and *Bacillus lysopersici* (Lin et al., 2015) were isolated from various samples and described. The genus *Bacillus* contains more than two hundred species with validly published names according to the LPSN website (List of prokaryotic strains with standing in nomenclature, http://www.bacterio.net/bacillus.html).

While investigating bacterial diversity in a pesticide wastewater sample, the standard serial-dilution plating technique was used to isolate strains on nutrient agar (NA; Hi-Media) and tryptic soy agar (TSA; Difco) at 30 °C for 3 days. A presumably novel strain (designated CC-LY275ᵀ) was selected for further phenotypic and phylogenetic characterization. Strain CC-LY275ᵀ was routinely grown aerobically on TSA at 30 °C for 2 days and stored at −80 °C in nutrient broth 2216 (NB; Hi-Media) supplemented with 30% (v/v) glycerol for long-term preservation. For taxonomic purposes, *Bacillus horneckiae* BCRC 80312ᵀ (Vaishampayan et al., 2010) and *Bacillus oceaniseudiminis* BCRC 80336ᵀ (Zhang et al., 2010) were used as reference strains for the comparison of phenotypic properties. All strains were grown on TSA at 30 °C for 2 days, unless specified otherwise. In order to clarify the taxonomic position of the novel strain, a polyphasic study including morphological and biochemical characteristics, phylogenetic characteristics of the 16S rRNA, polar lipid and cellular fatty acid composition was performed in comparison with reference strains of the genus *Bacillus*. The morphological, biochemical and phylogenetic characteristics of the novel bacterium was described according to the minimal standards (Logan et al., 2009; Tindall et al., 2010).

A commercial DNA extraction kit (MO BIO UltraClean) was used to extract the genomic DNA of CC-LY275ᵀ for 16S rRNA gene amplification and DNA G+C content determination. To obtain the almost complete 16S rRNA gene sequence of strain CC-LY275ᵀ, the PCR was performed with bacterial universal primers 1F and 9R (Edwards et al., 1989). Then, the PCR amplicon was ligated into a T&A cloning vector using a commercial cloning kit.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-LY275ᵀ is KR534504.

Two supplementary figures are available with the online Supplementary Material.
(Eastern Biotech) according to the manufacturer's instructions. The inserted 16S rRNA gene was sequenced using the M13-F and M13-R primers of the cloning kit. The quality of the resulting 16S rRNA gene sequence of strain CC-LY275\textsuperscript{T} was checked manually. Gene cycle sequencing was performed by using the Bigdye terminator kit (Heiner et al., 1998) and ABI 3730 Genetic Analyzer (Applied Biosystems). For the analysis of DNA G+C content, DNA samples were prepared and degraded enzymically into nucleosides as described by Mesbah et al. (1989). The nucleoside mixture obtained was then separated via HPLC [Hitachi L-2130 chromatograph equipped with a Hitachi L-2200 autosampler, Hitachi L-2455 Diode array detector, and a reverse-phase C18 column (Phenomenex Synergi Fusion-RP80 250 × 4.60 mm)].

The DNA fragments encoding 16S rRNA were assembled using the Vector NTI 9.0 software (IBI) and deposited in GenBank using sequin. The almost complete 16S rRNA sequences were retrieved from EzTaxon-e and GenBank and aligned by using the CLUSTAL X (1.83) program (Thompson et al., 1997). Phylogenetic trees were reconstructed by using 16S rRNA gene sequences with neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods and evaluated by bootstrap analyses (Felsenstein, 1985) after 1000 replications.

For the investigation of chemotaxonomic characteristics, strain CC-LY275\textsuperscript{T} and the reference strains were harvested at a similar physiological age given that the three strains showed similar growth kinetics. Polar lipids were extracted and analysed by two-dimensional TLC (Minnikin et al., 1984), and isoprenoid quinones were purified by the methods according to Minnikin et al. (1984) and analysed by HPLC as described by Collins (1985). The cell-wall diamino acid was determined from whole-cell hydrolysates (6 M HCl, 100 °C, 18 h) subjected to TLC on cellulose plates using the solvent system of Rhuland et al. (1955).

Fatty acid methyl esters (FAMEs) were prepared, separated and identified according to the standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI) (Sasser, 1990) by using a gas chromatograph (Agilent 7890A) fitted with a flame-ionization detector. For the extraction of FAMEs, strain CC-LY275\textsuperscript{T}, B. horneckiae BCRC 80312\textsuperscript{T} and B. oceani\textsuperscript{d}iminis BCRC 80336\textsuperscript{T} were cultured simultaneously on TSA for 48 h at 30 °C. Culture was harvested from the plate and subjected to saponification, methylation and extraction (Miller, 1982). Identification and comparison were made by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0).

Colonies were white, circular, raised and transparent after 2 days of incubation on TSA. Ellipsoidal spores developed subterminally in the cells (Fig. S1 available in the online Supplementary Material). Cell morphology was evaluated...
Fig. 1. Phylogenetic analysis of species of the genus *Bacillus* based on 16S rRNA gene sequences. Distances and clustering were performed by using the neighbour-joining method with the software package MEGA version 6. Filled circles indicate that the corresponding nodes were also recovered in the tree reconstructed on the basis of the maximum-likelihood algorithm. Bootstrap values (>50%) based on 1000 replications are listed as percentages at the branching points. Bar, 0.01 substitutions per nucleotide position.
by transmission electron microscopy (Fig. 2). Strain CC-LY275T could grow at 20–45 °C, pH ranging from pH 7.0 to 8.0, and was able to tolerate up to NaCl 6% (w/v) in tryptic soy broth. Strains CC-LY275T, B. horneckiae BCRC 80312T and B. oceanisediminis BCRC 80336T were positive for the utilization of Tween 40, Tween 80, acetic acid and pyruvic acid in the Biolog GP2 system; positive for arginine dihydrolase and urease in the API 20NE system; positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, z-chymotrypsin and naphthol-AS-BI-phosphohydrolase in the API ZYM system.

**Table 1. Polyphasic characterization of strain CC-LY275T and its closest phylogenetic relatives in the genus Bacillus**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>Reduction of nitrates (API 20NE)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Gelatin hydrolysis (API 20NE)</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>Utilization of:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>z-Cyclohextrin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>z-Ketovaleric acid</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Maltooltriose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>d-Ribose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Propionic acid</td>
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<td>-</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>l-Serine</td>
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<td>-</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>Assimilation of:</td>
<td></td>
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<td>d-Glucose</td>
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<td>N-Acetylgulcosamine</td>
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<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Potassium gluconate</td>
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<td>+</td>
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<td>Adipic acid</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>Malic acid</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Trisodium citrate</td>
<td>w</td>
<td>w</td>
<td>+</td>
<td>+</td>
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<td>Enzymes</td>
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<tr>
<td>Trypsin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>z-glucosidase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>37.9 ± 0.1</td>
<td>35.6–36.1</td>
<td>44.8</td>
<td>38.5</td>
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</tbody>
</table>

*Data in columns 2–4 were taken from Vaishampayan et al. (2010), Zhang et al. (2010) and Hong et al., (2012).*

Based on the distinct phylogenetic, phenotypic, biochemical and chemotaxonomic data provided, strain CC-LY275T fitted the description of the genus Bacillus and is proposed to represent a novel species, for which the name Bacillus formosensis sp. nov. is suggested.

**Description of Bacillus formosensis sp. nov.**

*Bacillus formosensis* [for.mo.sen’sis. N.L. masc. adj. formosensis of or pertaining to Formosa (Taiwan), the beautiful island].

Colonies are white, circular, raised and transparent after 2 days of incubation on TSA; colony size is about 1 mm.
The growth temperature ranges from 20 to 45 °C (optimum at 30 °C); grows at pH 7.0–8.0 (optimum at pH 8.0) and tolerates NaCl 6% (w/v). Gram-stain-positive, endospore-forming, aerobic, motile with lophotrichous flagella, rod-shaped (1.6–1.7 μm), catalase- and oxidase-positive. Utilizes numerous compounds as sole source of carbon, including β-cyclodextrin, mannann, Tween 40, Tween 80, D-ribose, acetic acid and pyruvic acid. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, α-chymotrypsin and naphthol-AS-BI-phosphohydrolase are positive in the API ZYM system. The major fatty acids are iso-C14:0, iso-C15:0 and anteiso-C15:0. The cell-wall diamino acid is meso-diaminopimelic acid. The polar lipid profile comprises diphosphatidylglycerol, an unidentified glycolipid, phosphatidylethanolamine, phosphatidylglycerol and an unidentified phospholipid. The predominant quinone system is menaquinone (MK-7).

The type strain is CC-LY275T (=BCRC 80443T=JCM 18448T), isolated from a pesticide wastewater sample in Taiwan. The DNA G+C content of the type strain is 37.9 ± 0.1 mol%.

Acknowledgements

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References


Table 2. Comparison of the cellular fatty acid contents of strain CC-LY275T and closely related species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>Saturated</td>
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<td></td>
<td>9.0</td>
<td>2.6</td>
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<tr>
<td>C16:0</td>
<td>1.2</td>
<td></td>
<td>5.7</td>
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<tr>
<td>C16:1</td>
<td>1.8</td>
<td>3.8</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Unsatuated</td>
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<tr>
<td>C15:0</td>
<td></td>
<td></td>
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<tr>
<td>C16:0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Branched</td>
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<tr>
<td>iso-C13:0</td>
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<tr>
<td>iso-C14:0</td>
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<td>iso-C15:0</td>
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<tr>
<td>iso-C16:0</td>
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<tr>
<td>iso-C16:1</td>
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<tr>
<td>iso-C17:0</td>
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<td>iso-C17:0</td>
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<tr>
<td>Summed feature 3</td>
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<tr>
<td>Summed feature 4</td>
<td></td>
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</tbody>
</table>

Strains: 1, CC-LY275T; 2, B. horneckiae BCRC 80312T; 3, B. oceanisediminis BCRC 80336T; 4, Bacillus eiseniae KCCM 90092T (data from Hong et al., 2012). Data are percentages of total fatty acids. TR, Contains traces (<1%); –, not detected. Summed feature 3, C16:1ω6c; summed feature 4, anteiso-C17:1B/iso-C17:1 I.


