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 oceani sediminis (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 diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. Cell-wall peptidoglycan contained meso-diaminopimelic acid; menaquinone (MK-7) was the predominant 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., 2015) 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 lyopersici (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 oceani sediminis 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.
GenBank using sequin. The almost complete 16S rRNA using the Vector NTI 9.0 software (IBI) and deposited in. The DNA fragments encoding 16S rRNA were assembled especially through 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 4 m 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 gene sequence (1541 nt) of strain CC-LY275 T was compared with those of all type strains representing species with validly published names using the 16S rRNA gene sequence database (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). Phylogenetic analysis was performed with MEGA 6 software (version 6.0; Tamura et al. 2013). Closely related 16S rRNA gene 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 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 T, B. horneckiae BCRC 80312 T and B. oceanisediminis BCRC 80336 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 database of the MIDI System (Sherlock version 6.0). Colony morphology, presence of flagella and morphology of the cells of strain CC-LY275 T was investigated using colonies/cells grown on TSA agar. Growth of strain CC-LY275 T was also tested on NA, marine agar (MA; Hi-Media) and R2A (BD Difco) agar. Growth of strain CC-LY275 T was tested using tryptic soy broth at different temperatures (4, 15, 20, 25, 30, 37, 40, 45 and 50 °C), NaCl concentrations (0–10 % in 1 % increments) and pH (4.0–10.0) at intervals of 1.0 pH units using the following buffer systems: pH 4.0–5.0, 0.1 M citric acid/0.1 M trisodium citrate; pH 6.0–8.0, 0.2 M Na2HPO4/0.2 M NaH2PO4; pH 9.0–10.0, 0.1 M NaHCO3/0.1 M Na2CO3). Gram staining was performed as described by Murray et al. (1994). Sporangial appearance was observed after mala-chite-green staining of strain CC-LY275 T, grown on TSA plates (supplemented with 5 mg MnSO4 l–1) for 1 week (Murray et al., 1994). Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H2O2 and oxidase activity was determined by using 1 % (v/v) N,N,N’,N’-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). Cell morphology was studied by transmission electron microscopy (JEOL JEM-1400); samples were stained with 0.2 % uranyl acetate and also observed by light microscopy (Zeiss model A3000). The following properties were tested for all strains in parallel under the same conditions. Additional enzymic activities, biochemical features and carbon source oxidation abilities of strain CC-LY275 T and the reference strains were determined by using the API ZYM and API 20NE kits (bioMérieux) and GP2 MicroPlate system (Biolog), respectively, according to the instructions of the manufacturers. During 16S rRNA gene sequence analysis, the pairwise comparison indicated that strain CC-LY275 T shared similarity with B. horneckiae 1P01SC T (97.1 %) and B. oceanisediminis H2 T (96.8 %), and other species showed lower levels of similarity (<96.8 %) to strain CC-LY275 T. Given several species delimitation thresholds for 16S rRNA gene sequence similarity, such as 98.65 % (Kim et al., 2014), 98.7–99.0 % (Stackebrandt & Ebers, 2006) and 98.2–99.0 % (Meier-Kolthoff et al., 2013), the similarity values obtained for CC-LY275 T with regard to the reference strains analysed were well below the reported thresholds. Strain CC-LY275 T should therefore be considered a representative of a putative novel species of the genus Bacillus. Phylogenetic trees were reconstructed by using 16S rRNA gene sequences with neighbour-joining, maximum-likelihood and maximum-parsimony methods. Regardless of different evolutional comparisons, similar topology was obtained in all phylogenetic trees, which indicates that the novel species represented by CC-LY275 T forms an individual cluster from B. horneckiae among other recognized species of the genus Bacillus (Fig. 1). 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 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, α-chymotrypsin and naphthol-AS-BI-phosphohydrolase in the API ZYM system. +, Positive; –, negative; w, weakly positive reaction; NA, no data available.

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

<table>
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<th>Characteristic</th>
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<th>4</th>
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<tr>
<td>Reduction of nitrates (API 20NE)</td>
<td>–</td>
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<td>Gelatin hydrolysis (API 20NE)</td>
<td>w</td>
<td>+</td>
<td>+</td>
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<td>Assimilation of:</td>
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<td>-d-Glucose</td>
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<td>-Maltose</td>
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<td>-Potassium gluconate</td>
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<td>-Trisodium citrate</td>
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<tr>
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<td>-α-glucosidase</td>
<td>–</td>
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<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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*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, chymotrypsin and naphthol-AS-BI-phosphohydrolase are positive in the API ZYM system. The major fatty acids are iso-C14:0, iso-C15:0 anteiso-c anteiso-C17:0 TR. 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%.

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### References


