**Virgibacillus oceani** sp. nov. isolated from ocean sediment

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A Gram-stain-positive, moderately halophilic, motile, strictly aerobic, endospore-forming, rod-shaped bacterium, strain MY11\(^T\), was isolated from a sediment sample collected from the Western Pacific. This isolate grew in the presence of 0.5–18 % (w/v) NaCl and at pH 6.0–10.0 and 15–45 °C; optimum growth was observed with 3.5 % (w/v) NaCl and at pH 8.0–9.0 and 35–37 °C. Strain MY11\(^T\) had menaquinone 7 (MK-7) as the predominant respiratory quinone and anteiso-C\(_{15:0}\) and anteiso-C\(_{17:0}\) as major fatty acids. Major polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content was 34.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequences confirmed that strain MY11\(^T\) was a member of the genus *Virgibacillus*, exhibiting sequence similarities of 95.3–97.6 % to the type strains of recognized *Virgibacillus* species. Strain MY11\(^T\) could be differentiated from recognized species of the genus *Virgibacillus* based on phenotypic characteristics, chemotaxonomic differences, phylogenetic analysis and DNA–DNA hybridization data. On the basis of the data presented, strain MY11\(^T\) is considered to represent a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus oceani* sp. nov. is proposed. The type strain is MY11\(^T\) (=LMG 28105\(^T\)=CGMCC 1.12754\(^T\)=MCCC 1A09973\(^T\)).

The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) with *Virgibacillus pantothenicus* as the type species, and the description was then emended by Heyman *et al.* (2003). At the time of writing, the genus *Virgibacillus* comprises 26 recognized species (http://www. bacterio.net/virgibacillus.html). Members of the genus *Virgibacillus* exist in many types of habitat such as deteriorated mural paintings (Heyrman *et al.*, 2003), permafrost (Niederberger *et al.*, 2009), waste wash-water (Quesada *et al.*, 2007) and salt lakes (Carrasco *et al.*, 2009; Chen *et al.*, 2008; Jeon *et al.*, 2009). Members of the genus are *Gram*-positive or *Gram*-variable, endospore-forming, motile rods and are characterized chemotaxonomically by having menaquinone 7 (MK-7) as the predominant respiratory quinone and anteiso-C\(_{15:0}\) as the common major fatty acid (Heyman *et al.*, 2003). Here, we describe the taxonomic properties of a moderately halophilic bacterial strain belonging to the genus *Virgibacillus* using a polyphasic approach.

**Abbreviations:** ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Virgibacillus oceani* MY11\(^T\) is KJ144820.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

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**Growth of strain MY11\(^T\) at various temperatures (4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C) was examined on MA. The pH range and the optimum pH for growth were determined in MB that was adjusted to pH 4–11 (in increments of 0.5 pH units). The pH of MB was adjusted prior to sterilization using the following buffers: 50 mM citric acid/sodium citrate buffer (pH 4.0–7.0), 50 mM KH\(_2\)PO\(_4\)/NaOH buffer (pH 6.0–8.0), 50 mM barbital.
sodium/HCl buffer (pH 7.0–9.5), 50 mM glycine/NaOH buffer (pH 8.5–10.5) and 50 mM NaHCO₃/NaOH buffer (pH 9.5–11.0). Verification of the pH values after autoclaving revealed only minor changes. Growth at various NaCl concentrations (0, 0.5, 1, 2, 3, 3.5, 5, 7, 10, 12, 15, 18, 20 and 25 %, w/v) was investigated on MA prepared according to the formula of the medium except for the addition of NaCl. Cell morphology were observed by phase-contrast microscopy (model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL), using cells grown on modified MA medium (per litre distilled water: 5 g peptone, 1 g yeast extract, 35 g NaCl, 20 g agar; pH 9.0) for 2–3 days at 37 °C. The reaction was carried out according to the classical Gram-staining procedure described by Doetsch (1981) and the non-staining KOH method as described by Gregersen (1978). Motility was studied by puncture inoculation in semisolid agar medium. H₂S production was determined by the methods described by Bruns et al. (2001). Oxidase activity was evaluated by oxidation of 1 % p-aminodimethylaniline oxalate and catalase activity was determined by measurements of bubble production after the application of 3 % (v/v) hydrogen peroxide solution. Nitrate reduction and hydrolysis of casein, starch, gelatin, urea, CM-cellulose and Tweens 20, 60 and 80 were determined as described by Tindall et al. (2007). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber for about 2 weeks. Further physiological and biochemical characteristics were determined using the API 20NE and API ZYM systems (bioMérieux) and Biolog GN2 MicroPlate panels, according to the instructions of the manufacturer. Virgibacillus carmonenensis DSM 14868ᵀ, Vigibacillus halodenitrificans DSM 10037ᵀ, Virgibacillus kkeensis DSM 17056ᵀ, Virgibacillus litoralis DSM 21085ᵀ, Virgibacillus necropolis DSM 14866ᵀ and Virgibacillus pantothenticus JCM 20334ᵀ were selected as reference strains and evaluated together under experimental conditions identical to those used for strain MY11ᵀ, in which V. pantothenticus JCM 20334ᵀ was obtained from the Japan Collection of Microorganisms (JCM) and the other strains from the German Collection of Microorganisms and Cell Cultures (DSMZ).

Cells of strain MY11ᵀ were Gram-stain-positive, aerobic, motile by means of peritrichous flagella, rod-shaped and approximately 0.3–0.6 × 1.1–1.8 μm, and occurred singly or in pairs after incubation for 2 days at 37 °C on modified MA medium (Fig. 1). Terminal spherical to ellipsoidal endospores were observed in swollen sporangia (Fig. 1). Colonies were circular, raised, opaque, smooth, cream in colour and 1–2 mm in diameter after incubation for 3 days at 37 °C on modified MA. Strain MY11ᵀ grew at 15–45 °C, at pH 6.0–10.0 and in the presence of 0.5–18 % (w/v) NaCl, with optimum growth at 35–37 °C, at pH 8.0–9.0, and with 3.5 % NaCl. Strain MY11ᵀ tested positive for activity of catalase and oxidase, nitrate reduction, and hydrolysis of gelatin, casein, and Tweens 20 and 60, but gave negative results for H₂S, indole and urease production, methyl red test, and hydrolysis of Tween 80, starch and CM-cellulose. Strain MY11ᵀ could be differentiated from its phylogenetic relatives on the basis of a number of phenotypic characteristics, including ranges of growth temperature, NaCl tolerance concentration, enzyme activity and acid production (Table 1). Detailed differential phenotypic properties of strain MY11ᵀ and related strains are listed in Table 1 and other characteristics are given in the species description.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene of strain MY11ᵀ were performed as described by Li et al. (2007). The nearly complete 16S rRNA gene sequence was obtained with primers 27F (5’-AGAGTTTGTATCTGGCTCAG-3’) and 1492R (5’-GGTTAATTGACGGTGATC-3’). The sequence was compared with 16S rRNA gene sequences of valid species from GenBank via the BLAST program and the EzTaxon-e server (Kim et al., 2012). All sequence alignments were analysed with the MEGA6 software package (Tamura et al., 2013). For maximum-likelihood (ML) analysis, the best fit model for nucleotide substitution was selected from 24 models using MEGA6 based on the minimum Bayesian information criterion value. The best model in this study was the Kimura two-parameter model with gamma-distributed rates plus invariant sites. The ML tree was built using the best fit model and nearest-neighbour interchange for the ML heuristic method. For neighbour-joining (NJ) analysis, the average pairwise Jukes-Cantor distance was chosen to determine whether the sequence data were suitable for estimating NJ trees. The average distance in this study was 0.045, a value suitable for making NJ trees (Nei & Kumar, 2000). The NJ tree was built using the maximum-composite-likelihood method, and the rate variation among sites was modelled with a gamma distribution (gamma parameter=0.1 in this study). For maximum-parsimony (MP) analysis, the subtree pruning-regrafting method was used with the default setting of 10 trees. All ML, NJ and MP trees were built with partial deletion of gaps, and reliability of the phylogenetic trees was

![Fig. 1. Cell morphology of strain MY11ᵀ. (a) Phase-contrast micrograph of sporangia and vegetative cells. Sporangia are swollen with terminal ellipsoidal or spherical spores. Bar, 10 μm. For sporulation, strain MY11ᵀ was incubated on MA medium at 28 °C for 10 days, and cells were then harvested and visualized. (b) Transmission electron micrograph of negatively stained cells. Bar, 1 μm.](Image)
The almost-complete 16S rRNA gene sequence (1525 bp) for strain MY11T was obtained. Using the EzTaxon-e database, strain MY11T showed highest 16S rRNA gene sequence similarity to *V. carmonensis* LMG 20964T (97.6%), followed by *V. necropolis* LMG 19488T (97.3%), *V. halodenitrificans* DSM 10037T (97.3%), *V. litoralis* JSM 089168T (97.1%) and *V. kekensis* YIM-kky16T (97.0%). The sequence similarities between strain MY11T and the other type strains of the genus *Virgibacillus* were less than 97.0%.

In the phylogenetic trees reconstructed using the NJ, ML and MP algorithms, strain MY11T fell within the radiation of the cluster comprising species of the genus *Virgibacillus* (Fig. 2; see also Figs S1 and S2, available in the online Supplementary Material). Subsequently, DNA–DNA hybridization was carried out as described by De Ley et al. (1970) incorporating the modifications described by Huss et al. (1983), using a Perkin-Elmer Lambda 35 spectrophotometer equipped with a Peltier temperature-controlling programmer. Strain MY11T showed relatively low DNA–DNA relatedness to *V. carmonensis* DSM 14868T (49.5%; reciprocal reaction, 45.4%), *V. necropolis* DSM 14866T (42.8%, reciprocal reaction, 45.3%), *V. halodenitrificans* DSM 10037T (48.1%; reciprocal reaction, 48.6%), *V. litoralis* DSM 21085T (40.3%; reciprocal reaction, 38.5%) and *V. kekensis* DSM 17056T (36.2%; reciprocal reaction, 31.8%). The values were well below the threshold value (70%) recommended by Wayne et al. (1987) for the definition of members of a species. The phylogenetic analysis results and DNA–DNA relatedness values described above indicated that the new isolate MY11T may represent a novel species of the genus *Virgibacillus*.

Table 1. Differential characteristics between strain MY11T and the type strains of closely related species of the genus *Virgibacillus*

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Colony colour</td>
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<td>Cream</td>
<td>Cream</td>
<td>Cream</td>
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<td>S, E</td>
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<td>Spore position</td>
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<td>–</td>
<td>–</td>
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<td>–</td>
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<td>Temperature range (°C)</td>
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<td>10–45</td>
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<td>10–40</td>
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<td>20% (w/v) NaCl</td>
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<td>+</td>
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<td>+</td>
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<td>α-Glucosidase</td>
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<td>w</td>
<td>+</td>
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<td>w</td>
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<td>w</td>
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<tr>
<td>D-Galactose</td>
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<td>w</td>
<td>–</td>
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<tr>
<td>D-Manose</td>
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<td>–</td>
<td>+</td>
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<td>Mannitol</td>
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<td>w</td>
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<td>N-Acetylglucosamine</td>
<td>+</td>
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<td>–</td>
<td>w</td>
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<td>–</td>
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<td>Asculin</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>w</td>
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<tr>
<td>Sucrose</td>
<td>w</td>
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<td>w</td>
<td>–</td>
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<td>Trehalose</td>
<td>w</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>w</td>
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<td>Xylitol</td>
<td>w</td>
<td>–</td>
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<tr>
<td>Gentiobiose</td>
<td>w</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>34.2</td>
<td>37.3</td>
<td>38.0–39.0</td>
<td>40.2</td>
<td>38.9</td>
<td>41.8</td>
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</table>

estimated using bootstrap values based on 1000 iterations. The almost-complete 16S rRNA gene sequence (1525 bp) for strain MY11T was obtained. Using the EzTaxon-e database, strain MY11T showed highest 16S rRNA gene sequence similarity to *V. carmonensis* LMG 20964T (97.6%), followed by *V. necropolis* LMG 19488T (97.3%), *V. halodenitrificans* DSM 10037T (97.3%), *V. litoralis* JSM 089168T (97.1%) and *V. kekensis* YIM-kky16T (97.0%). The sequence similarities between strain MY11T and the other type strains of the genus *Virgibacillus* were less than 97.0%.
The DNA G+C content was determined as described by Mesbah et al. (1989) using reversed-phase HPLC. The G+C content of strain MY11T was 34.2 mol%, which was slightly less than the values reported for recognized members of the genus *Virgibacillus* (36–43 mol%) (Heyrman et al., 2003).

For chemotaxonomic analyses, biomass was obtained from cultures grown on modified MA medium for 3–4 days at 37 °C. Cellular fatty acids were analysed according to the instructions of the Sherlock Microbial Identification System (MIDI, version 6). Isoprenoid quinones were analysed using HPLC as described by Collins (1985). Polar lipids were extracted according to the procedures described by Minnikin et al. (1984) and separated by two-dimensional TLC on plates of silica gel 60 F254 (Merck). Chloroform/methanol/water (65:25:4, by vol.) was used for the first dimension and chloroform/methanol/acetic acid/water (40:7:5:6:2, by vol.) for the second dimension, as described by Minnikin et al. (1977). Total lipids were detected by spraying the plate with 10 % ethanolic molybdophosphoric acid and phospholipids were detected by spraying with molybdenum blue reagent. Aminolipids and glycolipids were detected by spraying with ninhydrin and α-naphthol-sulphuric acid reagent, respectively. Furthermore, the identity of lipids including phosphatidylglycerol and other lipids was confirmed by comparing with previously reported relative migration under identical solvent conditions (Minnikin et al., 1984).

The fatty acid profile of strain MY11T was composed mainly of anteiso-C15 : 0 (65.0 %), anteiso-C17 : 0 (14.4 %), iso-C16 : 0 (9.5 %), iso-C14 : 0 (5.4 %) and iso-C15 : 0 (2.5 %). Although the major fatty acid profile of strain MY11T was very similar to those of closely related members of the genus *Virgibacillus*, the proportions and composition of some fatty acids distinguished the new isolate from related strains (Table S1). The major respiratory quinone of strain MY11T was MK-7, which corresponded to the characteristic quinone found in members of the genus *Virgibacillus*. The total polar lipid profile as shown by two-dimensional...
TLC suggested that strain MY11\(^T\) contained diphosphatidylglycerol, phosphatidylglycerol, four unidentified lipids and nine unidentified phospholipids (Fig. S3), which were also observed in other species of the genus Virgibacillus (Heyndrickx et al., 1999; Heyrman et al., 2003). Together, the chemotaxonomic results for strain MY11\(^T\) determined in the present study were consistent with the chemotaxonomic characteristics of the genus Virgibacillus.

Phylogenetic and chemotaxonomic characteristics suggested that strain MY11\(^T\) belonged to the genus Virgibacillus. Furthermore, the DNA–DNA relatedness between strain MY11\(^T\) and the type strains of recognized species of the genus Virgibacillus was low. Physiological and biochemical traits distinguished strain MY11\(^T\) from other Virgibacillus species (Table 1). Cumulatively, these data suggested that strain MY11\(^T\) should be considered as a representative of a novel species of the genus Virgibacillus, for which the name Virgibacillus oceani sp. nov. is proposed.

### Description of Virgibacillus oceani sp. nov.

*Virgibacillus oceani* (o.ce.a’ni. L. gen. n. oceani of the ocean). Cells are Gram-stain-positive, aerobic, motile by means of peritrichous flagella, rod-shaped and approximately 0.3–0.6 × 1.1–1.8 μm, and occur singly or in pairs after incubation for 2 days at 37 °C on modified MA. Terminal spherical to ellipsoidal endospores are observed in swollen sporangia. Colonies are circular, raised, opaque, smooth, cream in colour and 1–2 mm in diameter after incubation for 3 days at 37 °C on modified MA. Growth occurs at 15–45 °C, at pH 6.0–10.0 and with 0.5–18 % (w/v) NaCl with optimum growth at 35–37 °C, at pH 8.0–9.0 and with 3.5 % NaCl. Catalase- and oxidase-positive. Positive for nitrate reduction and hydrolysis of gelatin, casein, and Tweens 20 and 60. Negative for H₂S, indole and urease production, methyl red test, and hydrolysis of Tween 80, starch and CM-cellulose. In the API 20E strip, positive for tryptophan deaminase, gelatinase and Voges–Proskauer reaction, but negative for β-galactosidase, arginine dihydrolase, urease, lysine decarboxylase, ornithine decarboxylase, citrate utilization, and production of H₂S and indole. Acid is not produced from inositol, D-sorbitol, L-rhamnose, sucrose, amygdalin, L-arabinose, D-mannitol or melibiose. In the API ZYM strip, positive for activity of alkaline phosphatase, esterase (C4), naphthol-AS-BI-phosphohydrolase and esterase lipase (C8); weakly positive for activity of leucine arylamidase, valine arylamidase and x-chymotrypsin; negative for activity of lipase (C14), cystine arylamidase, trypsin, acid phosphatase, α-galactosidase, β-galactosidase, α-mannosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamine and β-fucosidase. In the API 50 CH strip, positive for acid production from asacchalin, D-fructose, D-mannose and N-acetylglucosamine; weakly positive for acid production from glycerol, erythritol, D-ribose, D-xyllose, D-xylose, D-galactose, D-glucose, cellobiose, maltose, sucrose, trehalose, xylitol and gentiobiose; negative for acid production from the remaining 32 substrates. The major fatty acids are anteiso-C₁₅:₀, anteiso-C₁₇:₀, iso-C₁₆:₀, iso-C₁₄:₀ and iso-C₁₅:₀. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, four unidentified lipids and nine unidentified phospholipids. The predominant menaquinone is MK-7.

The type strain, MY11\(^T\) (=LMG 28105\(^T\)=CGMCC 1.12754\(^T\)=MCCC 1A09973\(^T\)), was isolated from a sediment sample collected from the Western Pacific. The G+C content of the genomic DNA of the type strain is 34.2 mol%.

### Acknowledgements

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### References


carmonensis sp. nov., Virgibacillus necropolis sp. nov. and Virgibacillus picturae sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus Saltibacillus to Virgibacillus, as Virgibacillus marismortui comb. nov. and Virgibacillus salexigens comb. nov., and emended description of the genus Virgibacillus. Int J Syst Evol Microbiol 53, 501–511.


