INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Xu *et al.*, *Int J Syst Evol Microbiol*
DOI 10.1099/ijsem.0.002782

MICROBIOLOGY SOCIETY

Virgibacillus indicus sp. nov. and *Virgibacillus profundi* sp. nov, two moderately halophilic bacteria isolated from marine sediment by using microfluidic streak plates

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Abstract

Three Gram-variable, moderately halophilic, motile, endospore-forming rods, designated P2-C2^T, P3-H5^T and P3-B8, were isolated from marine sediment of the Southwest Indian Ocean by using the microfluidic streak plate method. Phylogeny based on 16S rRNA gene sequences showed that strains P2-C2^T and P3-H5^T formed a robust cluster within the genus *Virgibacillus* and exhibited 16S rRNA gene similarity levels of 95.3–96.8 and 94.9–96.3 % to the type strains of *Virgibacillus* species, respectively. The 16S rRNA gene similarity between P2-C2^T and P3-H5^T was 97.6 %. Strain P3-B8 has an identical 16S rRNA gene sequence to strain P3-H5^T. For all the novel strains, the predominant cellular fatty acids were *anteiso*-$C_{15:0}$ and *anteiso*-$C_{17:0}$, the main menaquinone was MK-7, and the polar lipid profiles contained diphosphatidylglycerol and phosphatidylglycerol. The genomic DNA G+C contents of strains P2-C2^T, P3-H5^T and P3-B8 were 38.3, 37.3 and 37.5 mol%, respectively. Combined data from phenotypic and genotypic studies demonstrated that strains P2-C2^T and P3-H5^T/P3-B8 are representatives of two different novel species of the genus *Virgibacillus*, for which the name *Virgibacillus indicus* sp. nov. and *Virgibacillus profundi* are proposed. The type strains are P2-C2^T (=CGMCC 1.16138^T=NBRC 113014^T) and P3-H5^T (=CGMCC 1.16139^T=NBRC 113015^T).

For over a century, microbiologists had been using agar plates to cultivate microbes from various environmental samples. Although we could modify the nutrients in the media and physical parameters to promote the growth of different microbes, we still fell short and could only successfully culture in the lab less than one percent of microbes of that we could see under a microscope or detect by using the molecular technique [1, 2]. This was described as 'The Great Plate Count Anomaly'. Here, to increase the rate of isolation of uncultured species, we employed the microfluidic streak plate (MSP) method for high-throughput microbial cell separation and cultivation in nanolitre-volume sessile droplets [3, 4]. This method enabled the isolation and identification of a number of putative novel species, among which strains P2-C2^T, P3-H5^{T}and P3-B8 from amarine sediment habitat were recognized as two novel species of the genus *Virgibacillus*.

The genus *Virgibacillus* was created by the reclassification of *Bacillus pantothenticus* [5] as *Virgibacillus pantothenticus* [6, 7], and the genus description was subsequently emended by Heyrman *et al.* [8]. At the time of writing, the genus comprised 35 recognized species, including the recently described *Virgibacillus flavescens* [9], *Virgibacillus kapii* [10], *Virgibacillus natechei* [11], *Virgibacillus oceani* [12], *Virgibacillus jeotgali* [13] and '*Virgibacillus ainsalahensis*' [14]. Members of the genus *Virgibacillus* are Gram-stain-positive or Gram-stain-variable, endospore-forming, motile rods and characterized chemotaxonomically by having *meso*-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan (except for *Virgibacillus arcticus* [15],

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Keywords: *Virgibacillus*; *Virgibacillus indicus* sp. nov.; *Virgibacillus profundi* sp. nov.; droplet microfluidics; microfluidic streak plate; polyphasic taxonomy.
Abbreviations: ANI, average nucleotide identity; CGMCC, China General Microbiological Culture Collection Center; HPLC, high-performance liquid chromatography; MA, ZoBell's marine agar 2216E; MB, ZoBell's marine broth 2216E; ML, maximum-likelihood; MP, maximum-parsimony; NA, nutrient agar; NJ, neighbour-joining; ONPG, o-nitrophenyl-β-D-galactopyranoside; TSA, trypticase soy agar.
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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains P2-C2^T, P3-H5^T and P3-B8 are KY800369, KY800370 and KY800371, respectively. The whole genome shotgun project of strain P2-C2^T , P3-H5^T and P3-B8 has been deposited in DDBJ/ENA/GenBank under the accession numbers NPMS00000000, NPOA00000000 and NPMW00000000, respectively. The versions described in this paper are versions NPMS01000000, NPOA01000000 and NPMW01000000, respectively.
One supplementary table and three supplementary figures are available with the online version of this article.

Virgibacillus necropolis [8] and *V. flavescens* [9], which have peptidoglycan types A1α, A4β and A4α, respectively), MK-7 as the predominant menaquinone, and *anteiso*-$C_{15:0}$ as the common major fatty acid [9, 16–18]. In this study, we report the characterization of two novel members of the genus *Virgibacillus* isolated from marine sediment of the Southwest Indian Ocean by using the MSP method.

Strains P2-C2^T, P3-H5^T and P3-B8 were isolated from marine sediments collected from the Southwest Indian Ridge, a mid-ocean ridge located along the floors of the Southwest Indian Ocean and Southeast Atlantic Ocean. The sample for strains P2-C2^T and P3-H5^T was located at 54.55891° S 34.94898° E at a depth of −1675.9 m, and the sample for strain P3-B8 was located at 54.6153° S 34.84631° E at a depth of −2335.7 m. The subsurface sediments (collected at 5 cm depth) were collected by a television-grabbing bucket in June 2016, and stored in aliquots at 4 °C for the cultivation study and frozen at −80 °C for long-term storage.

Sediment samples were pretreated by the dispersion and differential centrifugation method [19], and then appropriate dilutions of the treated sample solutions (with a modified M13 *Verrucomicrobium* broth, the DSMZ Medium 607) were subjected to high throughput cultivation using the MSP workflow as described below. First, a microfluidic device was used to generate monodisperse droplets of 4–6 nl volumes carrying single bacterial cells. The droplets were then written robotically as stationary sessile droplet arrays on the surface of a polystyrene petri dish prefilled with mineral oil. Next, the oil-immersed droplets were incubated at 25 °C and the humidity of 98–100 % for up to 5 months to allow single cells to revive and divide. During the cultivation, we visualized and recorded the growth of cells in the droplets using an inverted microscope every 1~2 weeks. Those droplets with microbial growth were individually collected using a capillary, and were transferred into a 96-well plate for further cultivation and characterization.

Three cream-coloured novel isolates of pure cultures were recognized and designated P2-C2^T, P3-H5^T and P3-B8. The strains were routinely cultured on MA (ZoBell's Marine Agar 2216E; 212185, BD Difco) or MB (ZoBell's Marine Broth 2216E; 279110, BD Difco) at 25 °C and stored as a suspension in glycerol (15 %, v/v) at −80 °C. *Virgibacillus halodenitrificans* CGMCC 1.8915^T [20, 21], *V. necropolis* CGMCC 1.6145^T and *V. oceani* CGMCC 1.12754^T were routinely grown on MA or MB at 25 °C and used as a reference strains. The reference strains were obtained from the China General Microbiological Culture Collection Centre (CGMCC), and evaluated together under experimental conditions identical to those used for strain P2-C2^T, P3-H5^T and P3-B8 for all the physiological, biochemical and chemotaxonomic tests in this study.

Template DNA for PCR was extracted from strains P2-C2^T, P3-H5^T and P3-B8 with a commercial genomic DNA purification kit (DN1112, Beijing Biomed Tech. Co.) following the instructions of the manufacturer. The 16S rRNA gene sequence was amplified by PCR with a pair of universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACggCTACCTTgTTACgACTT-3′) under the following conditions: initial denaturation at 95 °C for 5 min prior to the addition of rTaq DNA polymerase (TAP-201; Toyobo) followed by 35 cycles of 95 °C for 45 s, 57 °C for 45 s and 68 °C for 2 min, and a final extension cycle at 68 °C for 10 min. Sequencing of the amplified product was carried out using the dideoxy chain termination/cycle sequencing technology on an ABI 3730XL automated DNA sequencer, and the ABI Big Dye Terminator kit (version 3.1) was used as the sequencing kit (maintained and performed in Beijing Tsingke Biological Tech. Co.).

We also extracted and purified the genomic DNA of strains P2-C2^T, P3-H5^T and P3-B8 for whole-genome sequencing, following a modified SDS-based DNA extraction method. The DNA library was reconstructed using a PCR-free method. The genome was sequenced on the Illumina HiSeq2500 platform in 250 bp paired-end read mode (PE250). DNA library construction and sequencing were performed at the Beijing Allwegene Technology Co., Ltd. Quality control of reads was performed using an in-house program. The filtered reads were assembled by SOAPdenovo [22] (http://soap.genomics.org.cn/soapdenovo.html) to generate scaffolds. The genomes were annotated using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) [23].

Complete 16S rRNA gene sequences were obtained from the direct sequencing of PCR products and the annotation of draft genomes. The sequences were compared with 16S rRNA gene sequences of valid species from GenBank via the BLAST program and the EzBioCloud online server [24]. Multiple sequence alignments were performed using the MUSCLE program [25] integrated in the MEGA software package version 7 [26]. Gblocks 0.91b program [27] was then employed to remove the alignment noise. Phylogenetic trees were reconstructed by using the neighbour-joining (NJ) [28] and maximum-parsimony (MP) [29] algorithms in the MEGA version 7. The maximum-likelihood (ML) [30] algorithm was inferred by using the PhyML version 3.0 server (www.atgc-montpellier.fr/phyml) [31]. For the NJ and ML algorithms, genetic distances were calculated by Kimura's two-parameter model and the complete deletion option was used. The nearest-neighbour interchange was applied in the ML analysis. In MP analyses, subtree-pruning–regrafting was used and alignment gaps were not considered. The resultant tree topologies generated from the NJ method were evaluated by bootstrap analysis based on 1000 replicates, and from the ML and MP methods based on 100 replicates.

Complete 16S rRNA gene sequences (1570 nt) for strains P2-C2^T, P3-H5^T and P3-B8 were obtained. Using the EzBioCloud database, strain P2-C2^T showed highest 16S rRNA gene sequence similarity to *V. halodenitrificans* DSM 10037^T (96.8 %), followed by *V. oceani* MY11^T (96.8 %), *Ornithinibacillus contaminans* CCUG 53201^T (96.6 %), *Virgibacillus salaries* SA-Vb1^T (96.4 %) and *Virgibacillus*

carmonensis LMG 20964^T (96.4 %). The sequences similarities between strain P2-C2^T and the other type strains of the genus *Virgibacillus* were less than 96.3 %. Strains P3-H5^T and P3-B8 had an identical 16S rRNA gene sequence, with highest similarities (>96.0 %) to *O. contaminans* CCUG 53201^T (96.3 %), *V. necropolis* LMG 19488^T (96.2 %), *V. oceani* MY11^T (96.2 %) and *V. flavescens* S1-20^T (96.0 %). The 16S rRNA gene similarity between P2-C2^T and P3-H5^T was 97.6 %, which was calculated by the ChunLab's online pairwise aligner for taxonomy (www.ezbiocloud.net/tools/pairAlign). In the phylogenetic trees reconstructed using the NJ, MP and ML algorithms, strains P2-C2^T, P3-H5^T and P3-B8 fell within the radiation of the genus *Virgibacillus*, representing two separate phylotypes, phylotype P2-C2^T and phylotype P3-H5^T/P3-B8, which were highly coherent to each other (Fig. 1; see also Fig. S1, available in the version version of this article). The reconstructed phylogenies also indicated a weak relatedness of the novel strains to '*Virgibacillus ainsalahensis*' MerV, with 16S rRNA gene sequence similarity of only 95.0 and 95.5 % for strains P2-C2^T and P3-H5^T/P3-B8, respectively.

The DNA G+C contents of P2-C2^T, P3-H5^T and P3-B8 were calculated from their draft genome data. ChunLab's online calculator [32] (www.ezbiocloud.net/tools/ani) was used to determine the average nucleotide identity (ANI) values between novel strains and the closest reference type strains of *V. necropolis* LMG 19488^T (CP022437) and *V. halodenitrificans* JCM 12304^T (BAZS01000000), with genome data available in DDBJ/ENA/GenBank. The genomes for strains P2-C2^T, P3-H5^T, and P3-B8 are 3 916 161 bp, 4 362 047 bp, and 4 407 795 bp long (one chromosome, no plasmid for each strain) with G+C contents of 38.3, 37.3, and 37.5 mol%, respectively, which were within the range reported for the type strains of other species of the genus *Virgibacillus* [8]. The ANI value between the genomes of strain P2-C2^T and P3-H5^T was 78.7 %, which was well below the generally accepted 95–96 % threshold range for species demarcation [33, 34]. In addition to the ANI value that determined between P3-H5^T and P3-B8 was 100.0 %, we concluded that P2-C2^T and P3-H5^T/P3-B8 should represent two single genomic species. As the ANI values were only 71.6 and 72.1 % of P2-C2^T to *V. necropolis* and *V. halodenitrificans*, and only 72.4 and 72.5 % of P3-H5^T/P3-B8 to *V. necropolis* and *V. halodenitrificans*, we refrained from calculating the ANI values of P2-C2^T or P3-H5^T/P3-B8 to other *Virgibacillus* species.

Strains P2-C2^T, P3-H5^T and P3-B8 displayed a requirement of seawater for better growth, so all media in this study were supplemented with artificial sea salts (S9883, Sigma-Aldrich) to give a final concentration of 3.4 % (w/v), unless otherwise indicated. Cultural and morphological properties of these new strains were examined after incubation at room temperature (25 °C) for up to 14 days on MA, nutrient agar (NA; CM0001, Oxoid), R2A (218263, BD Difco) and trypticase soy agar (TSA; 211043, BD BBL). Cell morphology was examined by a light microscope (ECLIPSE Ti, Nikon) equipped with a 20×long working distance objective (model CFI Plan Fluor; Nikon) and fitted with a DS-Fi1 camera (Digital Sight Series; Nikon). The micro-morphology was determined by transmission electron microscopy (model JEM-1400; JEOL) of cells grown on MA at 25 °C for 3 days. Motility was examined by microscopy and by observing spreading growth on MA semi-solid agar media (containing 0.3 % agar). Gram-staining reaction was carried out by using the classical Hucker procedure. Aerobic growth on different media was assessed at 25 °C on MA, TSA and R2A. Growth under anaerobic conditions was examined after incubation for 5 days on MA supplemented with 10 mM KNO_3 at 25 °C in an anaerobic jar [containing Anaerocult A (1.13829, Merck) to produce anaerobic conditions]. Anaerobic growth was further confirmed by a deep stab inoculation into a semisolid agar sealed by a layer of petrolatum. Growth under microaerophilic conditions was investigated at 25 °C on MA after incubation in the microaerophilic atmosphere containing 8–10 % (v/v) carbon dioxide and 5–7 % (v/v) oxygen; this atmosphere was generated in sealed jars containing Anaerocult C (1.16275, Merck). Growth at 4, 10, 15, 20, 25, 28, 30, 37, 40, 42, 45 and 55 °C was assessed on MA and in MB (unshaken). Growth at pH 4–11 was determined at 25 °C in buffered MB (0.1 M acetic acid/acetate buffer for pH 4.0–6.0; 0.05 M KH_2PO_4/NaOH buffer for pH 6.0–8.0; 0.1 M Tris buffer for pH 7.0–11.0); growth was evaluated by turbidity of the culture medium. Salt tolerance was assessed at 25 °C on TSA and in TSB supplemented with 0–25 % (w/v) NaCl (0, 0.5, 1, 3, 5, 7, 10, 15, 20, and 25 %).

Catalase activity was determined by measurements of bubble production after the application of 10 % (v/v) hydrogen peroxide solution, and cytochrome c oxidase activity was evaluated by oxidation of 1 % (w/v) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. H_2S production was determined by inserting sterile lead acetate filter paper strips into the necks of MB tubes and examining for blackening after the same incubation periods. Nitrate reduction was tested by adding the Griess Reagent (first developed in 1879 by Peter Griess) and the diphenylamine reagent into cultures in a nitrate broth composed of MB supplemented with KNO_3 (0.1 %, w/v). The decomposition of adenine, L-tyrosine (0.5 %, w/v), hypoxanthine, xanthine, xylan (0.4 %, w/v), casein (1 %, w/v, skimmed milk) and guanine (0.05 %, w/v) was detected in MA after 7, 14, 28d at 25 °C, and clearing of the insoluble compounds from under and around areas of growth was scored as positive. The hydrolysis of Tweens 20, 40, 60 and 80 was examined in MA by the appearance of opacity after 7 and 14 d. Gelatin (0.4 %, w/v) and starch (1.0 %, w/v) degradation were detected in the same basal medium after 7d by flooding plates with tannic acid solution and Lugol's iodine solution, respectively. The hydrolysis of carboxymethyl-cellulose and pectin was tested by using the Congo red agar plate method. Chitinolytic activity was observed by the appearance of zones of clearing in colloidal chitin agar. The degradation of allantoin (0.33 %, w/v), urea (2 %, w/v) and hipputate (1 %, w/v) was investigated using the methods proposed by Gordon *et al.* [35]. The breakdown of aesculin

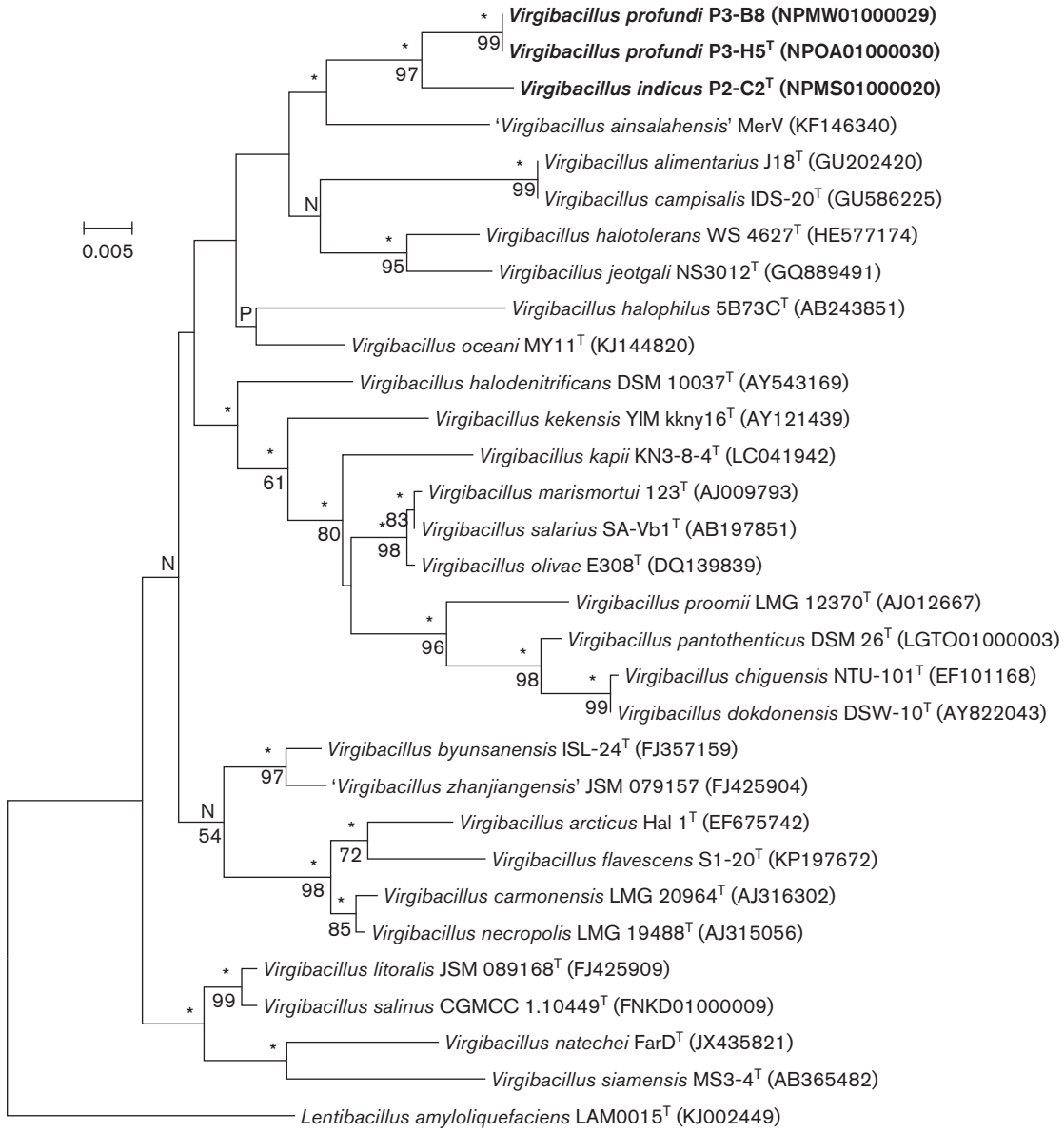

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequence data, showing the phylogenetic position of strains P2-C2^T, P3-H5^T and P3-B8 with respect to closely related members of the genus *Virgibacillus*. Bootstrap values (%) are based on 100 replicates and are shown for branches with >50 % support. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 0.5 % sequence divergence. N and P at tree nodes indicate branches that were also recovered using the neighbour-joining and maximum-parsimony tree-making algorithms, respectively. Asterisks indicate nodes that were recovered from all three algorithms above.

and arbutin (0.1 %, w/v) were determined by the method modified by Williams *et al.* [36]. The methyl red and Voges–Proskauer reactions, the o-nitrophenyl-β-D-galactopyranoside (ONPG) test, the indole production, and the amino acid-deaminase and dihydrolase tests were determined as described by Lányi [37]. Antibiotic resistances were tested by the disc diffusion method, performed with Oxoid disks on MA, spread with inoculum of tested strains. Plates were incubated at 28 °C for up to 1 week. The zone diameters were read as inhibition. Additionally, a series of chemical inhibitors were added at different concentrations to the MA plates, which were then inoculated with the tested strains to examining their susceptibility. Agar plates were kept at room temperature for up to two months, and growth of strains was read as resistance.

The assimilation of a number of carbohydrates as the sole carbon source (see the species description) at a final concentration of 0.2 % (w/v) was additionally tested at 25 °C water-washed agar culture in phosphate-buffered pH-neutral mineral medium supplemented with sea salts (3.4 %, w/v), NaCl (2 %, w/v), B vitamins and yeast extract

(0.02 %, w/v). Oxidative/fermentative metabolism of carbohydrates was determined on Hugh and Leifson's OF basal medium (1 % glucose or other carbohydrates, 0.2 % peptone, 0.1 % yeast extract, 0.5 % NaCl, 0.02 % K_2HPO_4, 0.008 % bromothymol blue, 3 % agar) supplemented with sea salts. In addition, physiological tests for determination of enzyme activities were performed by using the commercial API ZYM galleries of bioMérieux (incubated at 28 °C overnight). All tests were carried out simultaneously with studied strains and the reference strains.

Strains P2-C2^T and P3-H5^T/P3-B8 showed good growth on MA or NA, R2A and TSA supplemented with 3.4 % of artificial sea salts. P2-C2^T was tested susceptible to the following antibiotics (per disc): chloramphenicol (30 μg), rifampcin (5 μg), tetracycline (30 μg), and vancomycin (30 μg); but resistant to (per disc) ciprofloxacin (5 μg), erythromycin (15 μg), kanamycin (30 μg), lincomycin hydrochloride (2 μg) and streptomycin (10 μg). P2-C2^T was able to grow with chemical inhibitors of barium chloride (0.5 %), cobalt chloride (1.0 %) and zinc chloride (0.5 and 1.0 %), but not with potassium tellurite (1.0 %) and sodium azide (0.5 and 1.0 %). P3-H5^T/P3-B8 was susceptible to (per disc) chloramphenicol (30 μg), ciprofloxacin (5 μg), rifampcin (5 μg), tetracycline (30 μg), and vancomycin (30 μg), but resistant to (per disc) erythromycin (15 μg), kanamycin (30 μg), lincomycin hydrochloride (2 μg) and streptomycin (10 μg). Tests for ability of P3-H5^T/P3-B8 to grow on chemical inhibitors were positive for barium chloride (0.5 %) and zinc chloride (0.5 and 1.0 %), but negative for potassium tellurite (1.0 %) and sodium azide (0.5 and 1.0 %). The other morphological, physiological and biochemical characteristics of strains P2-C2^T, P3-H5^T and P3-B8 are given in the species description, and the features that differentiate the investigated strains from the reference strain are given in Table 1.

For chemotaxonomic characterization, strains P2-C2^T, P3-H5^T and the reference strains were cultured and evaluated together under identical experimental conditions. All the strains were cultivated in MB at 180 r.p.m. for 3 days at 25 °C (unless otherwise indicated), and cells were harvested by centrifugation and then washed twice in sterile saline. Whole-cell amino acids in whole-organism hydrolysates were prepared according to Lechevalier and Lechevalier [38] and analysed by standard thin-layer chromatography) and high-performance liquid chromatography (HPLC) procedures [39, 40]. Respiratory quinones were extracted and purified according to Collins *et al.* [41] and analysed by HPLC [42] using MK-7 from *V. halodenitrificans* CGMCC 1.8915^T as a reference. Polar lipid profiles were analysed according to the method modified from Minnikin *et al.* [43]. For fatty acid analysis, the three strains were grown on TSA supplemented with sea salts at 25 °C for 2–3 d for preparing biomass. Sufficient amount of bacterial cells of comparable physiological age of the three strains were harvested from the third streak-quadrant of the agar plates after cultivation under the applied conditions. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI, version 6.1) [44], using the database TSBA6 for calculation. Fatty acid analyses were carried out by the Identification Service of CGMCC.

Strains P2-C2^T, P3-H5^T and P3-B8 contained *meso*-diaminopimelic acid as the diagnostic amino acid in cell-wall peptidoglycan. The predominant menaquinone of strains P2-C2^T, P3-H5^T and P3-B8 was MK-7 (90.7, 87.6, and 88.6 %), which is in agreement with the genus description [8, 15, 18]. Minor amounts of MK-6 (4.3, 5.2, and 5.0 %) and MK-5 (5.0, 7.2, and 6.4 %) were also present. The polar lipid profile of strains P2-C2^T, P3-H5^T and P3-B8 contained diphosphatidylglycerol and phosphatidylglycerol, but no glycolipids and aminolipids were detected (Fig. S3). The major polar lipid components of strains P2-C2^T, P3-H5^T and P3-B8 (diphosphatidylglycerol and phosphatidylglycerol) were the same as those of the related species, *V. halodenitrificans*, *V. oceani* and *V. necropolis*. However, phosphatidylethanolamine as one of the major polar lipid components was found in other species of this genus, such as *Virgibacillus soli* [17], *Virgibacillus salarius* [45] and *Virgibacillus dokdonensis* [46].

The predominant cellular fatty acids (>5.0 % of total fatty acids) of strain P2-C2^T were *anteiso*-$C_{15:0}$ (57.0 %), *anteiso*-$C_{17:0}$ (25.2 %) and *iso*-$C_{16:0}$ (6.9 %). The main fatty acids (>5.0 %) for strain P3-H5^T were *anteiso*-$C_{15:0}$ (70.1 %), *anteiso*-$C_{17:0}$ (8.5 %) and *iso*-$C_{15:0}$ (5.7 %). Thus, the fatty acid profiles of strains P2-C2^T and P3-H5^T resembled those of other species of the genus *Virgibacillus* [8, 15, 18]. However, differences in the profiles obtained from the concurrent analyses clearly distinguished these two new isolates from each other, and from the selected reference species. Details of the fatty acid profiles of strains P2-C2^T, P3-H5^T and *V. halodenitrificans* CGMCC 1.8915^T are available in Table S1.

Data presented in this study demonstrate that strains P2-C2^T, P3-H5^T and P3-B8 represent two new members of the genus *Virgibacillus* and are distinguished from their phylogenetic neighbours and from each other by a combination of genotypic and phenotypic properties. Therefore, it is proposed that strains P2-C2^{T}and P3-H5^T represent two novel species of the genus *Virgibacillus*, for which the names *Virgibacillus indicus* sp. nov. and *Virgibacillus profundi* sp. nov. are proposed, respectively.

DESCRIPTION OF *VIRGIBACILLUS INDICUS* SP. NOV.

Virgibacillus indicus (in′di.cus. L. masc. adj. *indicus* of India, Indian, referring to the Indian Ocean, from where the strain was isolated).

Cells are Gram-stain-variable (young cells stain Gram-stain-positive) rods, 0.8–1.0×1.6–2.3 μm in size after 3 days at 25 °C on MA, occur singly or in pairs, and motile by peritrichous flagella (Fig. S2). Central or subterminal spherical to ellipsoidal endospores are observed in swollen sporangia. Colonies on

Table 1. Differential characteristics of strains P2-C2^T, P3-H5^T and P3-B8 isolated from marine sediments and the reference species within the genus *Virgibacillus*

Strains: 1, P2-C2^T; 2, P3-H5^T (identical results for strain P3-B8 unless indicated in parentheses); 3, *V. halodenitrificans* CGMCC 1.8915^T; 4, *V. oceani* MY11^T; 5, *V. necropolis* LMG 20964^T. +, Positive; –, negative; W, weakly positive; V, variable; ND, not determined. Data in columns 1–3 are from this study. Data marked with asterisks in columns 3, 4 and 5 are from Yoon *et al*. [21], Yin *et al*. [12], and Heyrman *et al*. [8]. All strains are rods, motile and positive for aerobic growth, catalase, oxidase and hydrolysis of casein and gelatin on agar plate tests, but negative for indole production, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, hydrolysis of carboxymethyl-cellulose, chitin, guanine, pectin, starch, tween 80, tyrosine and xylan, and fermentation of L-arabinose and glycogen. All strains are positive in API ZYM tests for alkaline phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for α-fucosidase, α-galactosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase and trypsin. All species contain MK-7 as the predominant menaquinone, and diphosphatidylglycerol and phosphatidylglycerol as the main polar lipids.

Characteristic	1	2	3	4	5
Gram staining	Gram-variable	Gram-variable	Gram-variable	Gram-positive	Gram-positive
Endospore formation	C, ST	ST	T, ST	T	T, ST, C
Anaerobic growth	+	+	+	–	–
Habitat	Marine sediment	Marine sediment	Solar saltern	Marine sediment	Mural paintings
Growth at:					
Temperature (°C)	4–42 (optimum 20–28)	4–30 (optimum 15–25)	1–45 (optimum 20–37)	15–45 (optimum 35–37)	10–40 (optimum 25–35)
pH value	6.0–10.0	6.5–9.0	6.0–9.0	6.0–10.0	6.0–9.0
NaCl (%)	0–15 (optimum 3–7)	0.5–15 (optimum 3–7)	0–25 (optimum 3–7)	0.5–20 (optimum 3–7)	0.5–20 (optimum 5–10)
Diagnostic diamino acid	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	L-Orn(L-Lys)-D-Glu*
Fatty acids	*anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, iso-$C_{16:0}$	*anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, iso-$C_{15:0}$	*anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, iso-$C_{16:0}$	*anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, iso-$C_{16:0}$, iso-$C_{14:0}$	*anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$
DNA G+C content (mol%)	38.3	37.3	37.5*	34.2*	37.3*
H_2S production	+	+	+	–	–
Nitrate reduction	+	+	+	+	V
Methyl red test	–	–	+	–	–
Voges–Proskauer reaction	–	–	–	+	+
ONPG test	–	+	+	–	+
Tryptophan deaminase	–	W	W	–	–
Decomposition of:					
Adenine	+	+	–	–	–
Aesculin	+	+	–	+	–
Allantoin	+	+	+	W	+
Arbutin	+	+	–	+	–
Hippurate	+	+	–	–	–
Hypoxanthine	+	W	–	–	–
Tween 20	–	–	–	+	W
Tween 60	–	–	–	+	W
Urea (urease)	–	+	+	W	+
Xanthine	W	+	–	–	–
Acid production from:					
D-Fructose	+	+	+	+	W
D-Galactose	+	W	+	W	–
N-acetyl-glucosamine	+	W	+	+	W
D-Glucose	+	W	+	W	W
Glycerol	+	+	+	W	W
myo-Inositol	W	–	–	–	–
Lactose	+	–	+	–	–
Maltose	+	–	+	W	W

Table 1. cont.

Characteristic	1	2	3	4	5
D-Mannitol	–	W	+	–	–
D-Mannose	+	W	+	+	W
Melibiose	W	–	–	–	–
L-Rhamnose	–	–	V	–	–
Sucrose	+	W (+)	+	W	–
Trehalose	+	–	+	W	W
D-Xylose	+	–	–	W	–
Enzyme assays (API ZYM):					
Acid phosphatase	W	W	+	–	–
α-Chymotrypsin	W	+	W	W	+
Cystine arylamidase	–	–	W	–	–
Esterase (C4)	W	W	W	+	+
Esterase lipase (C8)	W	W	W	+	–
β-Galactosidase	–	W (+)	+	–	+
N-acetyl-β-glucosaminidase	–	–	+	–	–
α-Glucosidase	+	–	+	–	+
Leucine arylamidase	–	– (+)	W	W	–
Valine arylamidase	–	–	–	W	–

+, Positive; —, negative; W, weak; V, variable; ND, not determined; C, central; T, terminal; ST, subterminal.

*Data from Yoon *et al.* [21], Yin *et al.* [12], and Heyrman *et al.* [8].

MA are circular to irregular with entire margins, low-raised, smooth, opaque, cream-coloured and 2–3 mm in diameter. Grows under aerobic and anaerobic conditions. Grows at 4–42 °C but not at 45 °C on MA. Grows at 25 °C in buffered MB at pH 6–10, on MA supplemented with 0.5–15 % (w/v) NaCl. Weak growth is observed on media without NaCl or sea salts supplemented. Positive for cytochrome c oxidase, catalase, H_2S production, nitrate reduction to nitrite, hydrolysis of casein (skimmed milk), gelatin, allantoin, hippurate, aesculin and arbutin, degradation of hypoxanthine, xanthine and adenine. Negative for the methyl red test, Voges–Proskauer reaction, ONPG test (for β-galactosidase), indole production, phenylalanine deaminase, tryptophan deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrolysis of starch, urea, carboxymethyl-cellulose, pectin, chitin and Tweens 20, 40, 60 and 80, degradation of tyrosine, guanine and xylan. Positive in manual tests for utilization of L-arabinose, D-fructose, D-glucose, lactose, sucrose and D-xylose as sole carbon sources. Positive for fermentation of *N*-acetylglucosamine, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannose, sucrose, trehalose and D-xylose. Negative for fermentation of L-arabinose, glycogen, D-mannitol and L-rhamnose. Weakly positive for fermentation of *myo*-inositol, and melibiose. In the API ZYM system supplemented with sea salts, positive for activities of alkaline phosphatase, α-glucosidase and naphthol-AS-BI-phosphohydrolase; weakly positive for acid phosphatase, α-chymotrypsin, esterase (C4), and esterase lipase (C8); and negative for cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, β-glucosidase, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, trypsin and valine arylamidase. Susceptible to chloramphenicol, rifampcin, tetracycline and vancomycin, but resistant to ciprofloxacin, erythromycin, kanamycin, lincomycin hydrochloride, and streptomycin. Tests for ability to grow on chemical inhibitors are positive for barium chloride, cobalt chloride and zinc chloride, but negative for potassium tellurite and sodium azide. The peptidoglycan contains *meso*-diaminopimelic acid. The predominant cellular fatty acids are *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$ and *iso*-$C_{16:0}$. The polar lipid profile contains diphosphatidylglycerol and phosphatidylglycerol. The predominant menaquinone is MK-7. Additional phenotypic properties are given in Table 1.

The type strain is P2-C2^T (=CGMCC 1.16138^T=NBRC 113014^T), which was isolated from marine sediment of the Southwest Indian Ocean. The genomic DNA G+C content of the type strain is 38.3 mol%.

DESCRIPTION OF *VIRGIBACILLUS PROFUNDI* SP. NOV.

Virgibacillus profundi (pro'fun.di. L. gen. n. *profundi* of/from the depths of the sea).

Cells are Gram-stain-variable (young cells stain Gram-stain-positive) rods, 0.7–0.9×1.5–3.5 µm in size after 3 days at 25 °C on MA, occur singly or in pairs, and have flagellar motility (Fig. S2). Subterminal spherical to ellipsoidal endospores are observed in swollen sporangia. Colonies on MA are circular to irregular with entire margins, convex, smooth, opaque, cream-coloured and 1–2 mm in diameter. Grows under aerobic and anaerobic conditions. Grows at 4–30 °C but not at 37 °C. Growth occurs at pH 6.5–9 and in the presence of 0.5–15.0 % (w/v) NaCl. No growth is observed on media without NaCl or sea salts supplemented. Positive for catalase, cytochrome c oxidase, H_2S production, nitrate reduction to nitrite, ONPG test, hydrolysis of abutin, aesculin, casein, allantoin, gelatin, hippurate and urea, and degradation of adenine and xanthine. Negative for indole production, methyl red test, Voges–Proskauer reaction, production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase and tryptophan deaminase, hydrolysis of carboxymethyl-cellulose, chitin, pectin, starch and Tweens 20, 40, 60 and 80, degradation of guanine, tyrosine, and xylan. Positive in manual tests for utilization of L-arabinose, D-fructose, D-glucose, lactose, sucrose, and D-xylose as sole carbon sources. Positive for fermentation of D-fructose and glycerol. Negative for fermentation of L-arabinose, glycogen, *myo*-inositol, lactose, maltose, melibiose, L-rhamnose, trehalose and D-xylose. Positive for alkaline phosphatase, α-chymotrypsin, β-galactosidase and naphthol-AS-BI-phosphohydrolase; weakly positive for acid phosphatase, esterase (C4), and esterase lipase (C8); but negative for cystine arylamidase, α-fucosidase, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, trypsin and valine arylamidase (API ZYM tests). Susceptible to chloramphenicol, ciprofloxacin, rifampcin, tetracycline and vancomycin, but resistant to erythromycin, kanamycin, lincomycin hydrochloride and streptomycin. Tests for ability to grow on chemical inhibitors are positive for barium chloride and zinc chloride, but negative for potassium tellurite and sodium azide. The peptidoglycan contains *meso*-diaminopimelic acid. The predominant cellular fatty acids are *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, and *iso*-$C_{15:0}$. The polar lipid profile contains diphosphatidylglycerol and phosphatidylglycerol. The predominant menaquinone is MK-7. Additional phenotypic properties are given in Table 1.

The type strain is P3-H5^T (=CGMCC 1.16139^T=NBRC 113015^T), which was isolated from marine sediment of the Southwest Indian Ocean. The genomic DNA G+C content of the type strain is 37.3 mol%.

Funding information
This study was supported by the Program of the China Ocean Mineral Resources R&D Association (No. DY135-B-02), the National Natural Science Foundation of China (31470142 and 31470221), the Strategic Priority Research Program of Chinese Academy of Sciences (XDB15040102), the Key Program of Frontier Sciences of Chinese Academy of Sciences (QYZDB-SSW-SMC008), and the Key Research Program of the Chinese Academy of Sciences (KFZD-SW-219-4).

Acknowledgements
The authors are indebted to Dr Hongcan Liu (CGMCC), Dr Lei Song (CGMCC) and Dr Jingnan Liang (IMCAS) for technical assistance with analysing the fatty acids, performing the API tests and operating the transmission electron microscope.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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