Kangiella shandongensis sp. nov., a novel species isolated from saltern in Yantai, China

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Abstract A Gram-stain-negative, wheat, rod-shaped, non-motile, non-spore forming, and facultatively anaerobic bacterium strain, designated as PI T, was isolated from saline silt samples collected in saltern in Yantai, Shandong, China. Growth was observed within the ranges 4–45 ºC (optimally at 33 ºC), pH 6.0–9.0 (optimally at pH 7.0) and 1.0–11.0% NaCl (optimally at 3.0%, w/v). Strain PI T showed highest 16S rRNA gene sequence similarity to Kangiella sediminilitoris BB-Mw22T (98.3%) and Kangiella taiwanensis KT1T (98.3%). The major cellular fatty acids (>10% of the total fatty acids) were iso-C15:0 (52.7%) and summed featured 9 (iso-C17:1ω9c/C16:1ω10-methyl, 11.8%). The major polar lipids identified were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylethanolamine and phosphatidylglycerol. The major respiratory isoprenoid quinone was Q-8. The G + C content of the genomic DNA was 45.8%. Average Nucleotide Identity values between whole genome sequences of strain PI T and next related type strains supported the novel species status. Based on physiological, biochemical, chemotaxonomic characteristics and genomic analysis, strain PI T is considered to represent a novel species within the genus Kangiella, for which the name Kangiella shandongensis sp. nov. is proposed. The type strain is PI T (= KCTC 82509T = MCCC 1K04352T).

Keywords 16S rRNA gene sequence · Kangiella shandongensis · Novel species · Polyphasic taxonomy

Abbreviations

AAI Average amino acid identity
ANI Average nucleotide identity
COG Cluster of orthologous groups
dDDH Digital DNA-DNA hybridization
DPG Diphosphatidylglycerol
GGDC Genome-to-genome distance calculator
HPLC High performance liquid chromatography
KCTC Korean collection for type cultures
KEGG Kyoto encyclopedia of genes and genomes
MA Marine agar 2216
MB Marine broth 2216
MEGA Molecular evolutionary genetics analysis
MCCC Marine culture collection of china
PE Phosphatidylethanolamine
PG Phosphatidylglycerol
PME Phosphatidylmonomethylethanolamine
VFDB Virulence factors of pathogenic bacteria

Introduction

The genus Kangiella, a member of the family Kangiellaceae, was first proposed by Yoon et al. (2004) with two type species Kangiella koreensis and Kangiella aquimarina (Yoon et al. 2004). At the time of writing, the family Kangiellaceae included the other genus Aliikangiella, which was first described by Wang et al. (2015). Over the last about seventeen years, the genus description was emended to accommodate eight further Kangiella species, Kangiella japonica (Romanenko et al. 2010), Kangiella spongi-cola (Ahn et al. 2011), Kangiella geojedonensis (Yoon et al. 2012), K. taiwanensis (Jean et al. 2012), Kangiella marina (Jean et al. 2012), K. sediminilitoris (Lee et al. 2013), Kangiella chungangensis (Kim et al. 2015) and Kangiella profundi (Xu et al. 2015) isolated from the aquatic environment in different regions. In general, all species of this genus are Gram-stain-negative, non-motile and rod-shaped bacteria, with Q-8 as the predominant isoprenoid quinone and iso-C₁₅:₀ as the predominant cellular fatty acid. (Yoon et al. 2004, 2012; Romanenko et al. 2010; Ahn et al. 2011; Jean et al. 2012; Lee et al. 2013; Kim et al. 2015; Xu et al. 2015).

In this study, a novel isolate strain PIT was isolated from saline silt samples collected in saltern in Yantai, Shandong, China, which presented most of features as above. The purpose of this study was to determine the exact taxonomic position of strain PIT by using a polyphonic approach, which included morphology, physiology, biochemical and detailed phylogenetic analysis based on the 16S rRNA gene sequence. The results of this study showed that strain PIT should be accommodated as the eleventh member of the genus Kangiella.

Materials and methods

Isolation procedure, maintenance and cultural conditions

Saline silt samples were collected from Muping saltern in Yantai, Shandong Province, China (121°40’20” E, 37°26’39” N) on the 23rd of March 2019 and the temperature of seawater was about 5 °C. Strain PIT was isolated by the dilution plating technique on marine agar 2216 (MA, Becton Dickin-son) at 33 °C. After incubating at 33 °C for 2 days, wheat colonies were observed and then picked from the plate and purified on MA. The obtained single colony was designated as strain PIT. Subcultivation was carried out on MA at 33 °C. K. sediminilitoris KCTC 23892 T and K. taiwanensis MCCC 1A06491 T which had the highest similarity with strain PIT and the type species of the genus Kangiella, K. aquimarina KCTC 12183 T were selected to be the reference strains. These three reference strains were all investigated for morphological, physiological and chemotaxonomic characterizations at the same conditions with strain PIT. The bacterial culture were preserved at −80 °C in sterile distilled water consisted of 1.0% NaCl (w/v) and 15.0% (v/v) glycerol.

16S rRNA gene phylogeny

Two universal primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) were used to amplify the 16S rRNA gene of strain PIT as described previously (Weisburg et al. 1991). The 16S rRNA gene sequence of strain PIT was checked using the BioEdit software (Hall 1999). NCBI BLAST and EzBioCloud were used to perform the comparison of 16S rRNA gene sequences (Yoon et al. 2017a). Based on all the strains with 16S rRNA sequence similarity above 95.0%, multiple alignments of their sequences were performed using Clustal_X version 1.83 with default settings (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Takahashi and Nei 2000) and maximum-parsimony (Fitch 1971) algorithms with the Kimura two-parameter model (Kimura 1980) in the MEGA (version 7.0) software (Kumar et al. 2016). Genome-based tree was
constructed using the TYGS server (https://tygs.dsmz.de/).

Morphological, physiological and biochemical analysis

Cell morphology and flagella were observed through an transmission electron microscope (Jem-1200; JEOL) after negative staining with 0.5% (w/v) uranyl acetate and air drying using cells incubated on MA at 33 °C for 3 days. Gliding motility was tested by preparing a light suspension of cells in seawater and then placing a drop on quarter-strength marine 2216 medium solidified with 1% agarose. After 24 h incubation, the inoculated area was covered with a glass coverslip and examined by oil-immersion phase-contrast microscopy (AX70; Olympus) (Bowman 2000). The growth temperature of strain PI T was determined on MA at 4, 10, 15, 20, 25, 30, 33, 37, 40, 42, 45 and 50 °C. The test of pH range for growth was performed in marine broth 2216 (MB, Becton Dickinson) by changing pH from pH 5.5 to 9.5 using the following buffer systems with a concentration of 20 mM: MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) and CAPSO (pH 9.0 and 9.5). Salt tolerance was tested in artificial seawater broth composed of 1.0 g L⁻¹ yeast extract, 5.0 g L⁻¹ peptone and NaCl with different concentrations (concentration range 0–12.0%, in increments of 0.5%, w/v) (Yang and Cho 2008). Gram-staining test was carried out according to the instructions by Smibert and Krieg (1994). After incubating on MA in the presence and absence of 0.1% (w/v) NaNO3 under anaerobic conditions at 33 °C for 3 days, the growth of strain PI T was observed to determine whether it could grow under anaerobic conditions. Antibiotic sensitivity test was performed on MA plates with discs (Tianhe) composed of various antibiotics (Penicillin, Ampicillin, Cefazolin, Amikacin, Gentamicin, Erythromycin, Norfloxacain, Ciprofloxacain, Bactrim and Chloramphenicol) at 33 °C for 3 days. The oxidation and fermentation test of carbohydrates were performed using cells grown for 2 days on MA through the Biolog GEN III Micro Plates and API 50CHB Fermentation Kit (bioMérieux) according to the manufacturer’s instructions. Other physiological tests were performed using API 20NE, API 20E, and API ZYM strips (bioMérieux).

Chemotaxonomic characterization

To determine the fatty acid compositions, strain PI T and reference strains were cultured in MB at 33 °C for 5 days approximately and the absorbance of biomass was measured by Absorbance Reader (CMax Plus) to ensure that the cells were at the mid-exponential phase. The compositions of fatty acids were analysed by gas chromatography, according to the standard Microbial Identification System (Sherlock version 6.1MIDI; TSBA6.0 database) (Sasser 1990). Respiratory quinone of strain PI T and reference strains were measured by using HPLC after grown in MB at 33 °C for 3 days (Kroppenstedt 1982). The polar lipids were separated by two-dimensional thin-layer chromatography (TLC) using chloroform/methanol/water (65:25:4, by vol) for the first dimension and chloroform/acetic acid/methanol/water (80:15:12:4, by vol) for the second dimension. Polar lipids were extracted and separated by two-dimensional TLC on Merck 5554 silica gel plates and spray reagents specific for each part were used to identify specific functional groups as described by Tindall et al. (2007). Molybdophosphoric acid (10%) was used to detect total lipid material.

Genome features

The software (SOAP, Spades and Abyss) were used to assemble sequence of genome and CISA was used for integration. The GeneMarkS (http://topaz.gatech.edu/) and IslandPath-DIOMB software were used to predict the coding genes and genomic island of strain PI T, respectively. The CPISPR gene cluster was forecasted using CRISPR digger. In order to analyse the antibiotic gene cluster of the strain PI T, the amino acid sequences of strain PI T were aligned with Comprehensive Antibiotic Research Database (CARD) to obtain the names of tolerated antibiotics and related drug-resistance genes. By using Diamond software, the amino acid sequences of strain PI T were compared with Kyoto Encyclopedia of Genes and Genomes (KEGG) and the genes of strain PI T were combined with their corresponding functional annotation information to analyse the genes involved in metabolic pathways (Kanehisa et al. 2016). In addition, Cluster of Orthologous Groups of proteins (COG) and Virulence Factors of Pathogenic Bacteria (VFDB) were also used to analyse the function classifications of
COG and virulence factors, respectively. The antiSMASH-4.0.2 program (Blin et al. 2017) was used to analyse secondary metabolic gene cluster. The DNA G + C content of strain PI^T was calculated from the genome sequence measured by Beijing Novogene Bioinformatics Technology Co, Ltd. (Beijing, PR China). The Average Amino Acid Identity (AAI) (Konstantinidis and Tiedje 2005; Rodriguez-R and Konstantinidis 2014a) calculator was carried out between strain PI^T and its closely related species (http://enve-omics.ce.gatech.edu/aai/). The Average Nucleotide Identity (ANI) and digital DNA-DNA Hybridization (dDDH) values were determined for the genome-based similarities, using the EzBioCloud integrated database (Yoon et al. 2017a), OrthoANIu algorithm of the EzGenome web service (Yoon et al. 2017b) and the Genome-to-Genome Distance Calculator (GGDC) version (Meier-Kolthof et al. 2013).

Results and discussion

Phylogenetic analysis

The complete 16S rRNA coding sequence of strain PI^T (1497 bp) was measured in this study. The phylogenetic tree based on 16S rRNA gene sequence using neighbour-joining method (Fig. 1), revealed strain PI^T formed a new phylectic line closely associated with the type strain of K. sediminilitoris BB-Mw22^T. The result of phylogenetic analysis according to neighbour-joining tree showed that strain PI^T belongs to the genus Kangiella (Fig. 1). The closest phylogenetic neighbours of strain PI^T were also depicted in the maximum-likelihood tree, which displayed the same tree topologies (Supplementary Fig. S1). The phylogenetic analysis of genome-based tree showed that strain PI^T had the highest similarity with K. sediminilitoris BB-Mw22^T and it belongs to the genus Kangiella (Fig. S2). On the basis of 16S rRNA gene sequence similarities and phylogenetic analysis, strain PI^T was most closely related to K. taiwanensis KT1^T (98.3%, sequence similarity) and K. sediminilitoris BB-Mw22^T (98.3%). It also had high similarity with other species of the genus Kangiella, indicating that strain PI^T belonged to this genus.

Morphological, physiological and biochemical analysis

Cells of strain PI^T were determined to be Gram-stain-negative, wheat, rod-shaped, non-spore-forming, oxidase and catalase-positive, which are consistent with these Kangiella species. Under the transmission electron microscope, strain PI^T was 0.4–0.5 μm in width, 1.0–2.0 μm in length (Supplementary Fig. S3). Growth of strain PI^T was observed over a pH range of 6.0–9.0 and NaCl concentrations range of 1.0–11.0%
(w/v), which distinguished from other species in the genus *Kangiella* (Table 1). Different from other *Kangiella* species, strain PT could grow at 4 °C. Strain PT was susceptible to erythromycin, trimethoprim-sulfamethoxazole and chloramphenicol, but resistant to ampicillin, amikacin, and gentamicin. Acid productions not observed with fermentation of glucose and other carbohydrates provided by the API 50CHB Fermentation Kit (bioMérieux). Other differential phenotypic characteristics were provided in Table 1. The differences above determined that strain PT was a distinct *Kangiella* species.

### Chemotaxonomic characteristics

As shown in Table 2, the main fatty acids of strain PT were identified as iso-C₁₅:₀ (52.7%) and Summed Feature 9 (iso-C₁₇:₁ω₉c/C₁₆:₀ 10-methyl, 11.8%), which were similar to *K. aquimarina* KCTC 12183 T and *K. taiwanensis* MCCC 1A06491 T. However, the amount of iso-C₁₅:₀, iso-C₁₁:₀ 3-OH and Summed Feature 9 (iso-C₁₇:₁ω₉c/C₁₆:₀ 10-methyl) could be used to different it from the reference strains. The predominant ubiquinone of strain PT was Q-8, which was consistent with other members of the genus *Kangiella* (Yoon et al. 2004, 2012; Romanenko et al. 2010; Ahn et al. 2011; Jean et al. 2012; Lee et al. 2013; Kim et al. 2015; Xu et al. 2015). The polar lipids included diphasphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME) and phosphatidylglycerol (PG) (Supplementary Fig. S4). PME was similar to polar lipids of the reference strains *K. sediminilitoris* (Lee et al. 2013) and *K. taiwanensis* (Jean et al. 2012).

### Table 1 Differential phenotypic characteristics of strain PT and other closely related members of the genus *Kangiella*. Strains: 1, PT; 2, *Kangiella aquimarina* KCTC 12183 T; 3, *Kangiella sediminilitoris* KCTC 23892 T; 4, *Kangiella taiwanensis* MCCC 1A06491 T. All data from this study except DNA G + C contents of the related strains, which were from the original species description: 2 (Yoon et al. 2004), 3 (Lee et al. 2013), 4 (Jean et al. 2012).

| Characteristic | 1 | 2 | 3 | 4 |
|---------------|---|---|---|---|
| **Growth range:** | | | | |
| Temperature range (optimum, °C) | 4–45 (33) | 10–48 (30–37) | 10–40 (30–37) | 15–40 (25–30) |
| NaCl range (optimum, %, w/v) | 1.0–11.0 (3.0) | 0.5–12.0 (2.0–3.0) | 0.5–13.0 (2.0–3.0) | 0.5–12.0 (2.0–5.0) |
| pH range (optimum, %) | 6.0–9.0 (7.0) | 5.5–8.0 (7.0–8.0) | 5.5–7.5 (7.0–7.5) | 7.0–9.0 (8.0) |
| Nitrate reduction | + | + | − | + |
| **Hydrolysis of:** | | | | |
| β-galactosidase | − | − | + | − |
| Gelatin | + | + | − | + |
| Cystine arylamidase | − | − | w | + |
| Trypsin | + | + | w | + |
| Esterase (C4) | w | + | + | + |
| Esterase lipase (C8) | w | + | + | + |
| α-Chymotrypsin | − | − | w | + |
| Acid phosphatase | + | + | − | − |
| Naphthol-AS-BI-phosphohydrolase | + | + | w | − |
| Alkaline phosphatase | + | + | + | − |
| Trypsin | + | + | w | + |
| **Acids from:** | | | | |
| Arabinose | + | − | − | − |
| Sucrose | − | − | + | − |
| **DNA G + C content (%)** | 45.8 | 44.0 | 48.9 | 43.9 |
Genome features of strain P1\textsuperscript{T} were depicted in Supplementary Table S1. The genome consisted of 2,653 genes with a total length of 2,809,662 bp, including 39 tRNA genes, 2 sRNA genes and 4 rRNA genes (one 5S, one 16S and two 23S rRNA genes). The N50 value was 308,341 bp and the N90 value was 96,858 bp. The depth of sequencing coverage was 549 x . The gene length distribution of strain P1\textsuperscript{T} was mainly concentrated in the range of 300–800 bp and more than 2000 bp (more than 200 bp), the detailed information was depicted in supplementary materials (Fig. S5). There were 2730 genes described by KEGG result annotation, in which 444 genes were related to metabolic pathways, 203 genes were related to biosynthesis of secondary metabolites, 149 genes were related to biosynthesis of antibiotics and 113 genes were related to microbial metabolism in diverse environments. VFDB analysis indicated that strain P1\textsuperscript{T} possessed virulence related genes, which involved in induction of apoptosis, proteolysis, immune system evasion, efflux pump, glycosylation of the flagellin, biofilm formation, cellular invasion, cell adhesion, resistance to serum killing and antibiotic susceptibility. The secondary metabolite of strain P1\textsuperscript{T} was predicted to be arylpolyene and siderophore, which was consisted 12 and 49 genes, respectively. The resistance genes of strain P1\textsuperscript{T} determined its resistance to fluoroquinolone, beta-lactam, isoniazid, aminoglycoside and glycopeptide (Details in Table S2). The COG of strain P1\textsuperscript{T} included 24 function classifications, for instance, those involved in posttranslational modification, protein turnover, chaperones, transcription, lipid transport and metabolism, coenzyme transport and metabolism, secondary metabolites biosynthesis, transport and catabolism (detailed in Fig. S6). The DNA G + C content of strain P1\textsuperscript{T} was 45.8%, which was within the range of DNA G + C contents for species of the genus Kangiella (43–49%) previously reported. AAI, ANI and dDDH values between strain P1\textsuperscript{T} and the related strains were shown in Table S3. The AAI value between strain P1\textsuperscript{T} and the most related type strain K. sediminilitoris was 83.9% (between 90 and 60%, which were probably the threshold value for species and genus, respectively) (Rodriguez-R and Konstantinidis 2014b). AAI value of 83.9% supported that strain P1\textsuperscript{T} represented a new species. The ANI values between strain P1\textsuperscript{T} and the reference strains of K. sediminilitoris, K. aquimarina and K. taiwanensis were 76.9%, 71.0% and 74.6%, respectively, which were far below the described ANI values (95.0–96.0%) for species classification (Kim et al. 2014). The dDDH values between strain P1\textsuperscript{T} and K. sediminilitoris, K. aquimarina and K. taiwanensis were 19.8%, 19.1% and 18.7%, respectively, which were lower than the prescribed species delineation standard of 70% (Meier-Kolthof et al. 2013). Based on the analysis of genome sequence, strain P1\textsuperscript{T} was proposed to accommodate the novel strains of genus Kangiella. A complete 16S rRNA gene sequence (1,497 bp) obtained from the genome sequence was 99.8% similarity with the clone sequence (1,525 bp) deposited in GenBank under accession number JACNML000000000.

| Fatty acid | 1 | 2 | 3 | 4 |
|-----------|---|---|---|---|
| Saturated fatty acids | | | | |
| C\textsubscript{14:0} | 1.4 | 1.7 | TR | 1.5 |
| C\textsubscript{16:0} | 4.9 | 1.2 | 4.4 | 2.7 |
| Branched fatty acids | | | | |
| iso-C\textsubscript{11:0} | 5.2 | 8.2 | 2.9 | 5.7 |
| iso-C\textsubscript{13:0} | TR | 3.8 | TR | 1.0 |
| iso-C\textsubscript{14:0} | TR | TR | TR | 2.3 |
| iso-C\textsubscript{15:0} | 52.7 | 53.0 | 49.4 | 47.7 |
| iso-C\textsubscript{15:1} F | 2.5 | 2.0 | 3.6 | 2.0 |
| Anteiso-C\textsubscript{15:0} | TR | 3.3 | TR | TR |
| iso-C\textsubscript{16:0} | TR | TR | 1.4 | 3.2 |
| iso-C\textsubscript{17:0} | 4.9 | 1.8 | 6.7 | 4.2 |
| iso-C\textsubscript{16:1} G | – | – | – | 2.9 |

Hydroxy fatty acids

| Fatty acid | 1 | 2 | 3 | 4 |
|-----------|---|---|---|---|
| iso-C\textsubscript{11:0} 3-OH | 8.6 | 14.0 | 6.2 | 11.2 |
| Summed Feature 1\textsuperscript{*} | TR | 2.5 | 1.3 | 1.8 |
| Summed Feature 9\textsuperscript{&} | 11.8 | 3.9 | 16.4 | 10.1 |

*Summed Feature 1, C\textsubscript{13:0} 3-OH and/or iso-C\textsubscript{15:1} H/i; & Summed Feature 9, iso-C\textsubscript{17:1} 09c and/or C\textsubscript{16:0} 10-methyl
Description of *Kangiella shandongensis* sp. nov

*Kangiella shandongensis* (shan.dong.en’sis. N.L. fem. adj. *shandongensis* pertaining to Shandong, where the type strain was isolated).

Cells (0.4–0.5 μm × 1.0–2.0 μm) are Gram-staining negative, facultatively anaerobic, non-motile, rod-shaped and non-spore-forming. The colonies are wheat, circular, flat, smooth, opaque and 1–2 mm in diameter after cultured on MA at 33 °C for 3 days. Growth occurs at 4–45 °C (optimum 33 °C), pH 6.0–9.0 (optimum 7.0) and 1.0–11.0% (w/v) NaCl (optimum 3.0%). The predominant ubiquinone is Q-8 and the major fatty acids are iso-C15:0 and Summed Feature 9 (iso-C17:1ω9c/ω6c C16:0 10-methyl). The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol monomethylethanolamine and phosphatidylglycerol. The DNA G + C content is 45.8%.

The type strain is PiT (= KCTC 82509 T = MCCC 1K04352 T), isolated from saline silt samples collected in saltern in Yantai, Shandong, China. The 16S rRNA gene and genome sequences of strain PiT were submitted to GenBank with accession numbers MW407073 and JACNML00000000 respectively.

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Author contributions LYP wrote the manuscript and performed the research. HNJ, XXL and YM performed isolation, deposition, and identification. LYP and SKG performed genome analysis. YXZ contributed to study design. YXZ and RY revised the paper. All authors read and approved the final manuscript.

Declarations

Conflict of interest All the authors have declared no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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