Kordiimonas lacus sp. nov., isolated from a ballast water tank, and emended description of the genus *Kordiimonas*

Xue-Wei Xu,^{1,2} Ying-Yi Huo,³ Xue-Dong Bai,^{3,4} Chun-Sheng Wang,^{1,2} Aharon Oren,⁵ Sui-Yan Li⁴ and Min Wu³

Correspondence

Xue-Wei Xu
xuxw@sio.org.cn

¹Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Hangzhou 310012, PR China

²Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, PR China

³College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

⁴School of Life Science and Engineering, Southwest Jiaotong University, Chengdu 610031, PR China

⁵Institute of Life Sciences, and the Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

A Gram-negative, motile, rod-shaped bacterial strain, designated S3-22^T, was isolated from a sediment sample collected from a ballast water tank of a commercial ship and subjected to a polyphasic taxonomic characterization. The isolate formed small, light-yellow, semi-translucent and circular colonies on solid complex media. The strain was oxidase- and catalase-positive and metabolized a large number of carbon sources. Chemotaxonomic analysis showed ubiquinone Q-10 as predominant respiratory quinone, phosphatidylglycerol and an unidentified glycolipid as major polar lipids and iso-C_{17:1}ω9c, iso-C_{15:0}, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{16:0}, iso-C_{17:0} and C_{18:1}ω7c as major fatty acids and the hydroxy fatty acids iso-C_{17:0} 3-OH and C_{16:0} 3-OH. The genomic DNA G+C content was 54.9 mol%. 16S rRNA gene sequence analysis revealed that the isolate has 96.1 % similarity to the type strain of *Kordiimonas gwangyangensis*, the sole described species within the order *Kordiimonadales*, and less than 91.0 % similarity to other recognized species. On the basis of phenotypic and genotypic data, strain S3-22^T represents a novel species of the genus *Kordiimonas*, for which the name *Kordiimonas lacus* sp. nov. is proposed, with the type strain S3-22^T (=CGMCC 1.9109^T =JCM 16261^T). An emended description of the genus *Kordiimonas* is also presented.

Ballast water is essential for ships to maintain stability and safety at sea when sailing without cargo. When the water is discharged, sediments and biofilm from the ship's ballast water tanks can release invasive aquatic species to coastal regions. Micro-organisms have been largely ignored in ballast water research (Drake *et al.*, 2007). The interesting environment of ballast water tanks has rarely been explored for the presence of novel species, and little is known about the potential significance of ship-mediated transfer of these micro-organisms. Here we present a polyphasic study describing a novel *Kordiimonas* strain isolated from a sediment sample collected from a ship's ballast water tank. The genus *Kordiimonas*, belonging to the order

Kordiimonadales within the class *Alphaproteobacteria*, was proposed by Kwon *et al.* (2005). At the time of writing, *Kordiimonas gwangyangensis* was the sole described species within the order *Kordiimonadales*.

The sediment sample was collected from the ballast water tank of a commercial ship arriving at Zhoushan, Zhejiang province, China, in November 2007, and was stored in darkness at 4 °C until micro-organisms were isolated in February 2008. Approximately 100 mg sediment subsample was incubated for 24 h in ZoBell medium, which contained (per l distilled water) 19.45 g NaCl, 8.8 g MgCl₂, 3.24 g Na₂SO₄, 1.8 g CaCl₂, 0.55 g KCl, 0.16 g NaHCO₃, 0.1 g ferric citrate pentahydrate, 0.08 g KBr, 34 mg SrCl₂, 22 mg H₃BO₃, 4.0 mg Na₂SiO₃, 2.4 mg NaF, 1.6 mg NH₄NO₃, 8.0 mg Na₃PO₄, 5.0 g peptone (BD) and 1.0 g yeast extract (BD), pH 7.4 (ZoBell, 1941). The liquid was plated on ZoBell agar plates using a tenfold dilution series.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S3-22^T is FJ847942.

Four supplementary figures and a supplementary table are available with the online version of this paper.

After 3 days of aerobic inoculation at 30 °C, a light-yellow colony, designated S3-22^T, was picked. The strain was purified by repeated restreaking; purity was confirmed by the uniformity of colony morphology. Unless otherwise stated, strain S3-22^T was maintained on marine agar 2216 (MA; BD) at 35 °C.

Optimal conditions for growth were determined by using marine broth 2216 (MB) with different NaCl concentrations (0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 7.5, 10.0, 15.0 and 20.0 %, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) or CAPSO (pH 9.0–10.5) to MB at concentrations of 25 mM. The temperature range for growth was determined by incubating at 4, 10, 15, 18, 25, 30, 37, 43 and 48 °C. Cell motility and morphology were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL).

Single-carbon-source assimilation tests were performed using basal medium (BM). The corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %), organic acid (0.1 %) or amino acid (0.1 %) was added to liquid medium. The BM (Mikhailov *et al.*, 2006) contained (l⁻¹ distilled water) 1.0 g NH₄Cl, 0.044 g K₂HPO₄, 0.028 g FeSO₄·7H₂O, 500 ml artificial seawater and 100 ml Tris/HCl (1 M, pH 7.5). The artificial seawater contained (l⁻¹ distilled water) 23.4 g NaCl, 24.6 g MgSO₄·7H₂O, 1.5 g KCl and 2.9 g CaCl₂. Acid production was tested using modified marine oxidation-fermentation (MOF) medium supplemented with 1.0 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Sensitivity to antimicrobial agents was determined on MA for at least 3 days. Oxidase and catalase activities, H₂S production, nitrate reduction and the ability to hydrolyse agar, casein, gelatin, starch and Tweens 20, 40, 60 and 80 were assessed according to Dong & Cai (2001). Additional enzyme activities and biochemical characteristics were determined using API 20E, API 20 NE and API ZYM kits (bioMérieux).

Quinones were extracted with chloroform/methanol (2:1, v/v) from freeze-dried cells. The extracts were filtered and evaporated to near dryness and then redissolved in chloroform/methanol (2:1, v/v). The fraction was analysed using an LC-MS spectrometer [Column Zorbax-ODS (4.6 × 250 mm) with methanol/2-propanol (4:1, v/v) as solvent at a flow rate of 1.5 ml min⁻¹ (Grossi *et al.*, 1992)]. The HPLC (Agilent 1200) was coupled to a Finnigan LCQ Deca XP Plus ion trap mass spectrometer (Thermo). Fatty acid methyl esters obtained from cells grown on MA for 48 h at 35 °C were analysed by GC/MS (Kuykendall *et al.*, 1988). Polar lipids were extracted using a chloroform/methanol system and separated by two-dimensional TLC using silica gel 60 F₂₅₄ aluminium-backed thin-layer plates (Merck) (Kates, 1986). The solvent systems chloroform/methanol/water (65:24:4, by vol.) in the first dimension and chloroform/glacial acetic acid/methanol/water (80:12:15:4, by vol.) in the second dimension were used. Separated components were visualized by treating the

plates with 10 % (w/v) molybdotetraphosphoric acid followed by heating at 150 °C for 5 min. Glycolipids were detected by spraying the plate with 0.5 % (w/v) α -naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1) following by heating at 120 °C for 5 min; phospholipids were detected with Zinzadze reagent. Genomic DNA was obtained by using the method described by Marmur (1961). Purified DNA was hydrolysed with P1 nuclease and nucleotides were dephosphorylated with calf intestine alkaline phosphatase; the DNA G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Mesbah & Whitman, 1989).

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). PCR products were cloned into vector pMD 19-T (TaKaRa) and then sequenced to determine the almost-complete sequence of the 16S rRNA gene. The sequence was compared with closely related sequences of reference organisms from the FASTA and EzTaxon service (Chun *et al.*, 2007). Similar sequences were aligned using the PHYDIT 3.1 program (Jeon *et al.*, 2005). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 4 program package (Tamura *et al.*, 2007) and the maximum-likelihood method (Felsenstein, 1981) with the PHYLIP 3.6a3 software package. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method.

Cells of strain S3-22^T were Gram-negative rods, approx. 0.5–0.8 μ m wide and 3.0–6.0 μ m long. Cells were motile by means of flagella (Supplementary Fig. S1, available in IJSEM Online). Strain S3-22^T was able to hydrolyse aesculin, gelatin, starch and Tweens 20, 40, 60 and 80 and to utilize more than 20 carbon sources including glycerol, glucose and L-glutamine. The results indicated that the isolate could grow well in seawater containing mixed soluble organic materials. Phenotypic characteristics of strain S3-22^T are given in the species description. A comparison of the phenotypic properties of strain S3-22^T and *K. gwangyangensis* JCM 12864^T is shown in Table 1.

The almost-complete 16S rRNA gene sequence (1419 nt) of strain S3-22^T showed the highest sequence similarity (96.1 %) to that of the type strain of *K. gwangyangensis* and less than 91.0 % sequence similarity to sequences from other recognized species. The 16S rRNA gene sequence divergence between strain S3-22^T and the type strain of *K. gwangyangensis* exceeded 3 %, an accepted value for the distinction of different genomic species (Stackebrandt & Goebel, 1994). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S3-22^T formed a clade adjacent to the type strain of *K. gwangyangensis* with a high bootstrap resampling value (100 % by the neighbour-joining method) (Fig. 1). Trees based on the maximum-parsimony and maximum-likelihood methods are available as Supplementary

Table 1. Differentiating characteristics between strain S3-22^T and *K. gwangyangensis* JCM 12864^T

Data were obtained in this study under identical growth conditions. Both strains are positive for catalase, oxidase and hydrolysis of starch and Tween 80 and negative for arginine dihydrolase, lysine and ornithine decarboxylases, tryptophan deaminase and urease. Neither strain produces H₂S or indole. For both strains, the following compounds are utilized as sole carbon and energy sources: acetate, L-alanine, L-arginine, L-asparagine, L-aspartate, cellobiose, glucose, L-glutamine, L-glutamate, glycerol, L-histidine, *myo*-inositol, malate, maltose, L-ornithine, D-salicin, L-serine, trehalose and L-tyrosine. Acid is produced by both strains from cellobiose, glucose, *myo*-inositol, maltose and trehalose. Both strains are susceptible to (μ g per disc, unless indicated) chloramphenicol (30), neomycin (30), novobiocin (30), penicillin G (10 IU), rifampicin (5) and streptomycin (10). In API ZYM tests, both strains are positive for acid and alkaline phosphatases, *N*-acetyl- β -glucosaminidase, α -chymotrypsin, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and trypsin. +, Positive; –, negative; w, weakly positive.

Characteristic	Strain S3-22 ^T	<i>K. gwangyangensis</i> JCM 12864 ^T
Utilization of:		
Gluconate	+	–
Malonate	w	–
Mannose	–	+
D-Phenylalanine	–	+
Acid production from mannose	–	+
Sensitivity to (μ g per disc):		
Nitrofurantoin (300)	+	–
Tobramycin (10)	+	–
API ZYM results		
Cystine arylamidase	–	+
Lipase (C14)	–	w
α -Mannosidase	–	+
DNA G + C content (mol%)	54.9	55.6*

*Kwon *et al.* (2005) reported a value of 39.3 mol%.

Figs S2 and S3. Glycolipid analysis indicated that strain S3-22^T and *K. gwangyangensis* JCM 12864^T contained the same four glycolipids (GL1–GL4) but that the latter possessed an additional glycolipid (GL5) (Supplementary Fig. S4). Phospholipid analysis revealed that both contained large amounts of phosphatidylglycerol (identification based on location on the TLC plate, the presence of phosphate and the absence of sugar moieties). This characteristic is shared with members of the genera *Caulobacter*, *Brevundimonas*, *Maricaulis* and *Hyphomonas*, which belong to the order *Caulobacterales*, the closest neighbour of the order *Kordiimonadales* in phylogenetic trees (Abraham *et al.*, 1999). An in-depth study of the structure of the polar lipids found in the genus *Kordiimonas* is recommended in view of the diversity of lipids in phylogenetically related groups (Abraham *et al.*, 1997, 1999; Poindexter, 2005), but is outside the scope of this paper. The major fatty acids of strain S3-22^T

were iso-C_{17:1} ω 9c, iso-C_{15:0}, C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH, C_{16:0}, iso-C_{17:0} and C_{18:1} ω 7c, with hydroxy fatty acids iso-C_{17:0} 3-OH and C_{16:0} 3-OH. Branched fatty acids are seldom found in alphaproteobacteria, but high contents of iso- and anteiso-branched fatty acids were reported in *K. gwangyangensis* (Kwon *et al.*, 2005) and in *Croceicoccus marinus* (Xu *et al.*, 2009). The content of iso-branched fatty acids of strain S3-22^T (56.3%) was less than that of *K. gwangyangensis* JCM 12864^T (68.1%) (Supplementary Table S1). Strain S3-22^T and *K. gwangyangensis* JCM 12864^T could be differentiated by different reactions in tests for utilization of gluconate, malonate, mannose and D-phenylalanine as sole carbon and energy sources. Further differences included formation of acid from mannose (negative for strain S3-22^T), sensitivity to nitrofurantoin and tobramycin for strain S3-22^T and several enzyme activities (Table 1).

Ubiquinone Q-10 is found in most members of the *Alphaproteobacteria* (Dadhwal *et al.*, 2009). LC-MS analysis revealed that the predominant quinone of strain S3-22^T and *K. gwangyangensis* JCM 12864^T was Q-10. The data from the control strains, *Escherichia coli* K-12 (Q-8) and *Zhangella mobilis* CGMCC 1.7002^T (Q-10), were in agreement with data from the literature. The G + C content of DNA of strain S3-22^T was 54.9 $\pm$ 0.3 mol%, as determined by HPLC. This value is much higher than that reported for *K. gwangyangensis* (39.3 mol%; Kwon *et al.*, 2005). We therefore tested *K. gwangyangensis* JCM 12864^T as well, and found a value of 55.6 $\pm$ 0.3 mol%. *E. coli* K-12, *Piscibacillus halophilus* DSM 21622^T, *Oceanobacillus oncorhynchi* subsp. *incaldanensis* DSM 16557^T and *Sphingomonas melonis* DSM 14444^T, included as controls, gave values of 47.6, 38.7, 35.5 and 67.1 mol%, respectively, being close to values reported in the literature. We therefore conclude that the respiratory quinone profile and the G + C content published previously for *K. gwangyangensis* may be incorrect.

Based on the phenotypic differentiation (Table 1) and genotypic data presented above, we consider that strain S3-22^T represents a novel species of the genus *Kordiimonas*, for which the name *Kordiimonas lacus* sp. nov. is proposed.

Emended description of *Kordiimonas* Kwon *et al.* 2005

In addition to the characteristics reported by Kwon *et al.* (2005), the following properties are observed. The G + C content of the DNA varies between 54 and 56 mol%. The predominant quinone is Q-10. The polar lipid profile consists of the major components phosphatidylglycerol and an unidentified glycolipid and trace amounts of three unidentified glycolipids. The major fatty acids are iso-C_{17:1} ω 9c, iso-C_{15:0} and C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH.

Description of *Kordiimonas lacus* sp. nov.

Kordiimonas lacus (la'cus. L. gen. n. *lacus* of a lake or any large body of water, of a tank, referring to the isolation of the type strain from a ballast water tank).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain S3-22^T and related taxa. Bootstrap values are percentages based on 1000 replicates; only values >70 % are shown. Bar, 0.01 substitutions per nucleotide position. Filled circles indicate nodes recovered with bootstrap values >70 % in the maximum-parsimony tree. Trees based on the maximum-parsimony and maximum-likelihood methods are available as Supplementary Figs S2 and S3.

Gram-negative and motile by means of flagella. Cells are straight to slightly curved rods (0.5–0.8 µm wide and 3.0–6.0 µm long) with rounded ends. Colonies on MA are 1–2 mm in diameter, smooth, circular with regular borders and light-yellow after 72 h. Growth is observed at 0.5–10.0 % (w/v) NaCl with optimum growth at 2.0–3.0 %. The pH and temperature ranges for growth are pH 6.0–9.0 and 10–43 °C (optimum growth at pH 7.0 and 30–37 °C). Positive for oxidase and catalase. Aesculin, gelatin, starch and Tweens 20, 40, 60 and 80 are hydrolysed. Agar and casein are not hydrolysed. H₂S is produced from thiosulfate. Voges–Proskauer reaction is positive. Negative for arginine dihydrolase, glucose fermentation, indole production, lecithinase, lysine and ornithine decarboxylases, ONPG (*o*-nitrophenyl β-D-galactopyranoside) test, tryptophan deaminase and urease. The following constitutive enzyme activities are detected in API ZYM tests: acid and alkaline phosphatases, *N*-acetyl-β-glucosaminidase, α-chymotrypsin, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase. Cystine arylamidase, α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, lipase (C14) and α-mannosidase activities are not observed. The following compounds are used as sole carbon and energy sources: acetate, L-alanine, L-arginine, L-asparagine, L-aspartate, cellobiose, L-cysteine, gluconate, glucose, L-glutamate, L-glutamine, glycerol, L-histidine, *myo*-inositol, lactate, malate, maltose, L-ornithine, D-salicin, L-serine, trehalose and L-valine. Acid is produced from cellobiose, glucose, *myo*-inositol, maltose and trehalose. Susceptible to (µg per disc unless otherwise stated) bacitracin (0.04 IU), cefoxitin (30), chloramphenicol (30), neomycin (30), nitrofurantoin (300), novobiocin (30), penicillin G (10 IU), rifampicin (5), streptomycin (10) and tobramycin (10), but not susceptible to amoxicillin (10), ampicillin (10), carbenicillin (100), cefotaxime (30), erythromycin (15), kanamycin (30), nystatin (100),

polymyxin B (300 IU) or tetracycline (10). The predominant quinone is Q-10. The major polar lipids are phosphatidylglycerol and an unidentified glycolipid. Trace amounts of three unidentified glycolipids and 11 unidentified lipids are detected. The major fatty acids are iso-C_{17:1}ω₉c, iso-C_{15:0}, C_{16:1}ω₇c and/or iso-C_{15:0} 2-OH, C_{16:0}, iso-C_{17:0} and C_{18:1}ω₇c; the major hydroxy fatty acids are iso-C_{17:0} 3-OH and C_{16:0} 3-OH. The DNA G + C content of the type strain is 54.9 mol% (HPLC).

The type strain, S3-22^T (=CGMCC 1.9109^T =JCM 16261^T), was isolated from a sediment sample collected from a ballast water tank.

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