

Woeseia oceani gen. nov., sp. nov., a chemoheterotrophic member of the order *Chromatiales*, and proposal of *Woeseiaceae* fam. nov.

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A novel Gram-stain-negative, rods or bent rods, facultatively anaerobic, oxidase-negative and catalase-positive bacterium, designated XK5^T, was isolated from coastal sediment from Xiaoshi Island, Weihai, China. Optimal growth occurred at 28–35 °C (range 8–42 °C) and pH 7.0–8.0 (range pH 6.0–9.0) with 1–3 % (w/v) NaCl (range 0.5–8 %). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain XK5^T was 92.1 % similar to the type strain of *Thioalkalivibrio thiocyanodenitrificans*, 91.9 % to the type strain of *Thioalkalivibrio sulfidiphilus* and 91.8 % to the type strain of *Thioalkalivibrio denitrificans*; similarity to other species was less than 91 %. The isolate and closely related environmental clones formed a novel family level clade in the order *Chromatiales*. The polar lipid profile of the novel isolate consisted of phosphatidylethanolamine, phosphatidylglycerol and some other unknown phospholipids, aminolipids and lipids. Major cellular fatty acids were iso-C_{17:1ω9c} and iso-C_{15:0} and the main respiratory lipoquinone was Q-8. The DNA G + C content of strain XK5^T was 59.3 mol%. Comparative analysis of 16S rRNA gene sequences and characterization indicated that strain XK5^T represents a novel species of a new genus within a novel family of the order *Chromatiales*, for which the name *Woeseia oceani* gen. nov., sp. nov. is proposed. The type strain of *Woeseia oceani* is XK5^T (=ATCC BAA-2615^T=CICC 10905^T). In addition, a novel family name, *Woeseiaceae* fam. nov., is proposed to accommodate the genus *Woeseia*.

In the past, the taxonomy of bacteria was entirely based on simple phenotypic characteristics (Imhoff, 2005), which aroused a long-time dispute about the taxonomic position of the bacteria now classified as the *Ectothiorhodospiraceae* since their discovery by Pelsh (1936). Members of the *Ectothiorhodospiraceae* were previously treated as a genus of the *Chromatiaceae*. Until recently, with the application of phylogenetic taxonomy of 16S rRNA, the *Ectothiorhodospiraceae* was separated as a family distinct from the family *Chromatiaceae* (Imhoff, 1984). At the time of writing, the order *Chromatiales* contains five families, according to the *List of Prokaryotic Names with Standing in Nomenclature* (LPSN; Euzéby, 1997), and members of the order are famous for their various types of metabolism, such as photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic. Most phototrophic bacteria in the order *Chromatiales* can utilize

reduced sulfur compounds as electron donors, which forms highly refractile globules of elemental sulfur; the family *Chromatiaceae* deposit the sulfur globules inside the cells while the *Ectothiorhodospiraceae* outside (Pfennig & Trüper, 1974). In this study, a novel chemoheterotrophic bacterium that had significant differences with the members of the *Ectothiorhodospiraceae*, the closest relatives of the novel isolate, is reported. Based on the phenotypic and chemotaxonomic analysis, and integrating the results of phylogenetic trees based on 16S rRNA gene sequences, we concluded that the novel strain might represent a novel family of the *Chromatiales*.

During the course of studying the diversity of a marine microbial community, a novel chemoheterotrophic, facultatively anaerobic, brown, non-motile, Gram-negative strain, XK5^T, was isolated from marine sediment from Xiaoshi Island, Weihai, China (37° 31' 36" N 122° 00' 58" E) on 2216E agar (HopeBio). The sample was serially diluted to 10⁻⁶ with sterilized seawater and 0.1 ml aliquots of each dilution were spread onto 2216E agar. Plates were incubated at 28 °C for 5 days, and strain XK5^T was isolated and stored at -80 °C in sterile 15 % (v/v) glycerol supplemented with 1 % (v/v) NaCl.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain XK5^T is KM034745.

Two supplementary figures are available with the online Supplementary Material.

Genomic DNA of strain XK5^T was obtained from 36 h-old cultures on 2216E agar using a genomic DNA extraction kit (TakaraBio). The 16S rRNA gene of strain XK5^T was amplified from the genomic DNA by PCR with universal primers 27F and 1492R (Lane, 1991). PCR products were purified using a PCR product purification kit (Tiangen) and then ligated to the pGM-T vector (Tiangen) according to the manufacturer's instructions. Sequencing was performed by Life Biotechnology (Shanghai) using universal primers T7 and SP6. To identify the taxonomic status of strain XK5^T, a near complete sequence (1476 bp) was obtained and submitted to the GenBank/EMBL/DDBJ databases; and similar sequences were searched using the BLAST algorithm. The EzTaxon server (<http://ezbiocloud.net/eztaxon>; Kim *et al.*, 2012) was used to achieve the similarity values of sequences. The 16S rRNA gene sequence of strain XK5^T was aligned using SINA online (Pruesse *et al.*, 2012) with removing bases remaining unaligned at the ends. The aligned sequence was imported into the database of the Living Tree Project (Yarza *et al.*, 2008) release 119 using ARB software package (Ludwig *et al.*, 2004). Close phylogenetic relatives of strain XK5^T were found and marked using ARB, and then a positional filter of 50 % conservation based marked sequences was calculated and applied. The phylogenetic tree was reconstructed with the neighbour-joining method with Jukes–Cantor correction, and the maximum-likelihood tree reconstructed using RAxML with the GTRGAMMA model was combined with the neighbour-joining tree. All members of the family *Ectothiorhodospiraceae* were included in the tree and the members of the family *Sneathiellaceae* were used to root the tree.

The almost-complete 16S rRNA gene sequence (1476 bp) was obtained from strain XK5^T and BLAST searches revealed that the novel isolate had highest similarity to *Thioalkalivibrio thiocyanodenitrificans* DSM 16954^T (92.1 %; Sorokin *et al.* 2004), followed by two other members of the same genus, *Thioalkalivibrio sulfidiphilus* NCCB 100376^T (91.9 %; Sorokin *et al.* 2012) and *Thioalkalivibrio denitrificans* DSM 13742^T (91.8 %; Sorokin *et al.* 2001). Except for the above three strains, other published strains showed <91.0 % sequence similarity with strain XK5^T. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, the novel strain and some closely related environmental clones formed a separate clade out of the family *Ectothiorhodospiraceae* within the order *Chromatiales* (Fig. 1), which indicated it was a family level taxon of the *Chromatiales*. The maximum-likelihood phylogenetic tree was similar and both of them supported our conclusion that the novel strain represented a novel family within the *Chromatiales*.

The morphological and physiological features of strain XK5^T were examined after incubation at 33 °C for 1–2 days on 2216E agar. The morphology of colonies was observed on 2216E agar after incubation for 2–4 days at 33 °C. Motility was examined according to the hanging-drop method. A transmission electron microscope

(Jem-1200; JEOL) was used to observe the cell size and morphology, which was supplemented by light microscopy (Ci-L; Nikon). Gram-staining was carried out as described by Murray *et al.* (1994). To test the temperature range for growth, inoculated 2216E agar plates were incubated at 4, 8, 15, 20, 25, 28, 30, 33, 35, 37, 42 and 45 °C, and growth conditions were recorded every 6 h. NaCl tolerance was tested with modified 2216E agar with seawater replaced by artificial seawater containing different concentration of NaCl (0, 0.5 and 1–10 %, in 1 % increments, w/v), and growth conditions were also recorded every 6 h. To test the effect of pH on growth, 2216E liquid medium (HopeBio) was adjusted to different pH with different buffers, MES (for pH 5.5–6.0), PIPES (for pH 6.5, 7.0), HEPES (for pH 7.5–8.0), Tricine (for pH 8.5) and CAPSO (for pH 9.0–9.5), each at a concentration of 20 mM. The OD₆₀₀ of the cultures were measured after incubation for 36 h at 33 °C. Anaerobic growth was determined by placing inoculated 2216E agar with or without 0.1 % (w/v) KNO₃ in an anaerobic jar. For the nitrate-reducing test, 2216E liquid medium supplemented with 0.1 % (v/v) nitrate in test tubes was used. The inoculated test tubes were placed in aerobic and anaerobic conditions at 33 °C for 2 days. Uninoculated test tubes served as control groups. To evaluate the sulfur oxidation ability of strain XK5^T, 200 mg l⁻¹ Na₂S was added to 2216E broth. After incubation, the sulfate, sulfite, thiosulfate and elemental sulfur were tested by ion chromatography or HPLC. Catalase activity was detected by the production of bubbles after the addition of a drop of 3 % (v/v) H₂O₂, and oxidase activity was determined by using a bioMérieux oxidase reagent kit. Hydrolysis of starch, cellulose, lipid and algin was tested on 2216E agar supplemented with 0.2 % (w/v) soluble starch, 0.5 % (w/v) CM-cellulose, 1 % (v/v) Tween 80 and 2 % (w/v) sodium alginate, respectively (Cowan & Steel, 1965). Antibiotic sensitivity was assessed as described by the Clinical and Laboratory Standards Institute (CLSI, 2012): a cell suspension (McFarland standard 0.5) was swabbed over the surface of 2216E agar plates to create a uniform lawn before aseptic placement of antibiotic discs onto the agar surface. Inoculated plates were incubated at 33 °C for up to 4 days. Tests for other physiological and biochemical characteristics were determined with API 20E and API ZYM strips (bioMérieux), according to the manufacturer's instructions, except that the suspension was replaced by sterilized 3 % (w/v) NaCl solution. The novel isolate was further tested for its ability to oxidize different compounds using Biolog GEN III according to the manufacturer's instructions. Tests of acid production from carbohydrates were performed using the API 50CH fermentation kit (bioMérieux) according to the manufacturer's instructions. The salinity of the 50CHB/E medium and the Biolog medium was adjusted to 3 % by adding autoclaved 30 % (w/v) NaCl solution before inoculation. The results of API strips and Biolog plates were recorded every 6 h after incubation at 33 °C. All the API and Biolog tests were performed in duplicate, with appropriate positive and negative controls.

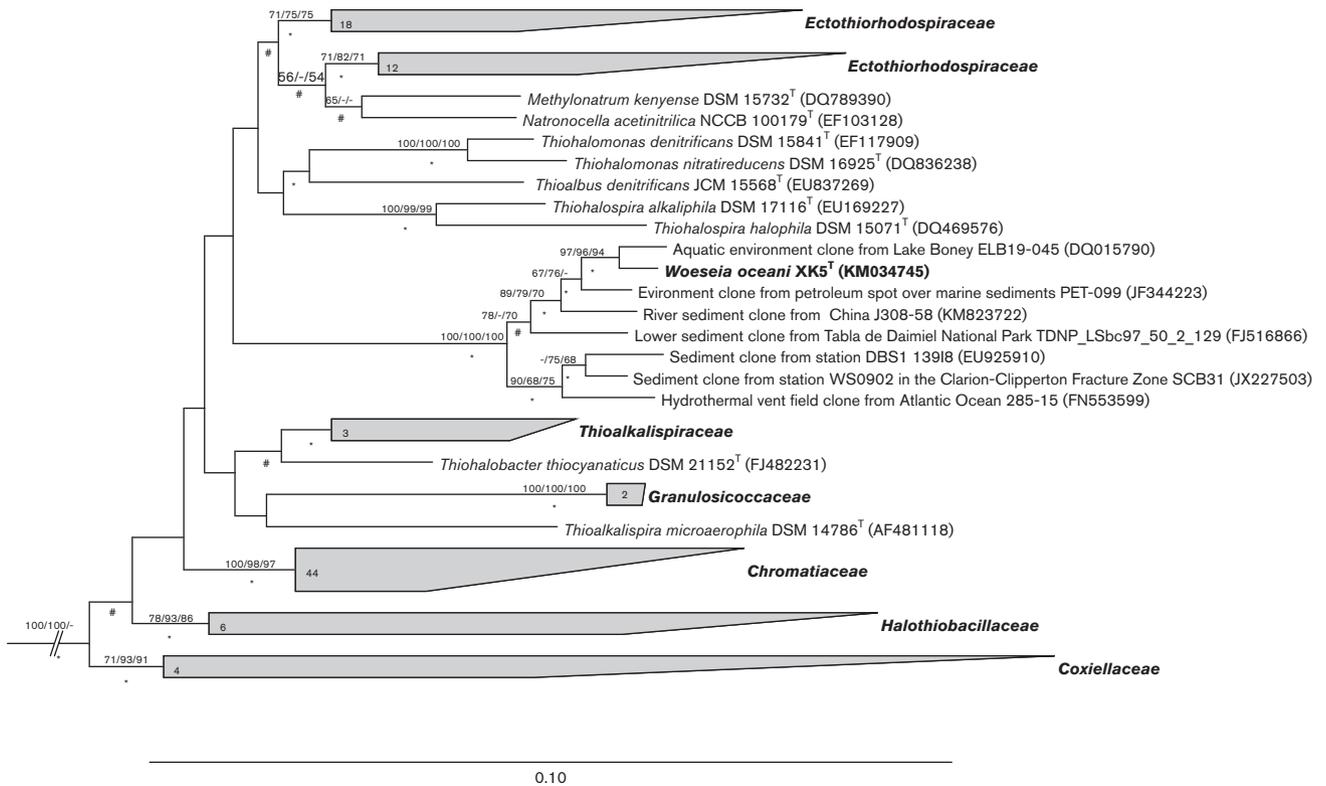


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain XK5^T and members of the order *Chromatiales* and some related unclassified *Gammaproteobacteria*. The database of LTP release 119 was used, and the family *Sneathiellaceae* was used to root the tree (not shown). Bootstrap values (>50 %) of neighbour-joining (1000 replications)/maximum-likelihood (100 replications)/maximum-parsimony (100 replications) methods are shown at the nodes. Asterisks and hash symbols indicate that the corresponding nodes were recovered reproducibly by all treeing methods or by two treeing methods, respectively. Bar, 0.1 substitutions per nucleotide position.

Strain XK5^T formed circular, viscid, brownish colonies, 1 mm in diameter, with entire and transparent edges after incubation for 2 days at 33 °C. The colour of the colonies would deepen to brown if the culture time was extended to 4 days. Brown or brownish colonies were only observed in some members of the genus *Thioalkalivibrio*, while yellow or red were more common for the close relatives (Table 1). Cells of strain XK5^T were non-motile and no flagellum was observed by transmission electron microscopy, however, all other close relatives showed motility with single polar, single subpolar, single bipolar or monopolar tuft flagella except some members of the genus *Thioalkalivibrio*. Electron micrographs (Fig. S1, available in the online Supplementary Material) showed that the cells of strain XK5^T were rods or bent rods, 0.3–0.5 × 0.8–2.5 µm in size. In contrast, cells of the genus *Thiorhodospira* were vibrio- or spiral-shaped and much longer; other close relatives were also different from the novel strain in size and shape (Table 1). The differences in morphology between strain XK5^T and its close relatives are rather obvious except that they are all Gram-negative, which supports our conclusion that strain XK5^T may represent a novel taxon.

Optimal growth occurred at 28–35 °C (range 8–42 °C) and pH 7.0–8.0 (range pH 6.0–9.0) with 1–3 % NaCl (range 0.5–8.0 %, v/v). However, most members of the family *Ectothiorhodospiraceae*, the closest related family of strain XK5^T, are halophilic or alkaliphilic. Considering the optimal pH and salinity, the novel strain can also be easily distinguished from its close relatives: the optimal pH for growth of members of the genera *Thioalkalivibrio*, *Natronocella*, *Thiorhodospira* and *Thiohalospira* is greater than 8.0, except for the type strain of *Thiohalospira halophila*, and the optimal concentration of Na⁺ for the genus *Thiohalospira* is 2–3 M. The sulfur oxidation ability of strain XK5^T was not observed. Strain XK5^T formed visible colonies on 2216E agar with or without 0.1 % (v/v) KNO₃ in the anaerobic jar, but it could not reduce nitrate in aerobic or anaerobic conditions. Among the related genera, the genus *Natronocella* showed facultatively anaerobic growth but could reduce nitrate; some members of the genus *Thioalkalivibrio* also could grow in aerobic or anaerobic conditions; however, the genus *Thiorhodospira* showed anaerobic growth while the genus *Thiohalospira* was microaerobic. In addition, the differences of metabolism between

Table 1. Differential characteristics of strain XK5^T and other related genera

Genera: 1, *Woeseia* gen. nov. (XK5^T; data from this study); 2, *Thioalkalivibrio* (Banciu *et al.*, 2004; Sorokin *et al.*, 2001, 2002a, b, 2003, 2004, 2012); 3, *Natronocella* (Sorokin *et al.*, 2007); 4, *Thiorhodospira* (Bryantseva *et al.*, 1999, 2010); 5, *Thiohalospira* (Sorokin *et al.*, 2008). –, Trace amount or not detected; ND, no data available.

Characteristic	1	2	3	4	5
Colony colour	Brownish to brown	White, yellow, reddish, brownish, orange-brown	Yellow	Brownish-red, red	Yellow
Morphology	Rods to bent rods	Coccioid or rods to spiral	Rods	Vibrio to spiral	Spiral
Cell size (μm)	0.3–0.5 \times 0.8–2.5	0.3–1.0 \times 0.8–8.0	0.4–0.5 \times 1.5–4.0	3.0–4.0 \times 7.0–20.0	0.4–0.5 \times 2.0–8.0
Flagella	None	Single polar/none	Single polar/subpolar	Monopolar tuft	Single bipolar/single polar
Nitrate reduction	Negative	Variable	Positive	ND	ND
O ₂ Metabolism	Facultatively anaerobic	Variable	Facultatively anaerobic	Anaerobic	Microaerobic
Metabolism	Chemoheterotrophic	Chemolithoautotrophic	Chemoheterotrophic	Photoheterotrophic	Chemolithoautotrophic
Optimal conditions for growth					
pH	7.0–8.0	8.0–10.2	9.5–9.8	8.5–9.5	7.3–8.5
Na ⁺ (M)	0.2–0.5	0.4–2	0.6	0.1–0.2	2–3
Major fatty acid (%)					
iso-C _{15:0}	19.0	–	–	–	–
C _{16:0}	11.6	18.2–29.3	13.0	18.1	Major
C _{18:0}	–	1.9–7.5	–	3.7	Major
iso-C _{17:1ω9c}	19.3	–	–	–	–
C _{18:1ω7c}	4.5	16.8–64.6	66.0	52.9	–
Major quinone	Q8	Q8	ND	ND	ND
DNA G+C content (mol%)	59.3	61.0–66.9	50.6–51.5	56.0–57.4	65.6–67.0

strain XK5^T and its close relatives are also significant. Autotrophic species can utilize reduced sulfur or light as energy sources and inorganic carbon as carbon sources, for example, members of the genera *Thioalkalivibrio* and *Thiohalospira*; the genus *Thiorhodospira* can also utilize light as an energy source, but it can utilize both inorganic and organic carbon as carbon sources (Bryantseva *et al.*, 1999). Although both strain XK5^T and the genus *Natronocella* are chemoheterotrophic, the genus *Natronocella* (Sorokin *et al.*, 2007) can utilize acetonitrile as a carbon, energy and nitrogen source while strain XK5^T utilizes peptone and yeast powder. Characteristics that can differentiate strain XK5^T from related genera are displayed in Table 1. In addition to the features mentioned above, strain XK5^T was weakly positive for catalase activity but negative for oxidase activity and hydrolysis of starch, lipid, align and cellulose. The positive result for gelatinase was the only positive reaction that occurred in the API 20E tests. According to API ZYM kits, cells were positive for alkaline phosphatase, esterase lipase (C4), leucine arylamidase, valine arylamidase, trypsin and chymotrypsin. Nine and three positive reactions were observed in Biolog and 50CH tests, respectively, the details are given in the species description.

Cells cultured on 2216E agar at 33 °C for 36 h were used to determine fatty acid composition. Fatty acids were saponified, methylated and extracted using the standard protocol of the Sherlock Microbial Identification System (MIDI) version 6.1 equipped with an Agilent model 6890N gas chromatograph. Peaks were automatically integrated and fatty acid names and percentages were calculated using the MIS standard software with the database TSBA 40. Polar lipids were separated by two-dimensional silica gel TLC. Total lipids were detected using molybdatophosphoric acid and specific spray reagents were used to detect defined functional groups; full details are given in Tindall *et al.* (2007). Both of the above procedures were carried out by the Identification Service of the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Respiratory lipoquinones were extracted from 300 mg freeze-dried cell material using the method described by Tindall (1990a, b). The lipoquinone extracts were separated into different classes by TLC on silica gel, removed from the plate and analysed further by HPLC.

The major fatty acids (relative amount >1 %) in strain XK5^T were iso-C_{17:1 ω 9c} (19.3 %), iso-C_{15:0} (19.0 %),

summed feature 3 ($C_{16:1\omega7c}/C_{16:1\omega6c}$; 11.9 %), $C_{16:0}$ (11.6 %), iso- $C_{16:0}$ (9.4 %), $C_{18:1\omega7c}$ (4.5 %), iso- $C_{17:0}$ (4.1 %), iso- $C_{14:0}$ (2.8 %), iso- $C_{11:0}$ 3-OH (2.4 %), iso- $C_{13:0}$ 3-OH (2.2 %), iso- $C_{11:0}$ (1.7 %), iso- $C_{10:0}$ (1.5 %) and $C_{14:0}$ (1.0 %). The components of fatty acids of the novel strain were significantly different from the close relatives. Unsaturated $C_{18:1\omega7c}$ and saturated $C_{16:0}$ were the major fatty acids of the genera *Thioalkalivibrio* (Sorokin *et al.*, 2012), *Natronocella* (Sorokin *et al.*, 2007) and *Thiorhodospira* (Bryantseva *et al.*, 2010), which accounted for 50–70 % of the total. In contrast, the total of these two fatty acids was 16.1 % in strain XK5^T (Table 1). The predominant fatty acids of strain XK5^T, iso- $C_{15:0}$ and iso- $C_{17:1\omega9c}$, were rare in relatives of the novel strain. 10-methyl $C_{16:0}$, $C_{16:0}$ and $C_{18:0}$ constituted more than 85 % of the total of the genus *Thiohalospira* (Sorokin *et al.*, 2008). Strain XK5^T could be easily distinguished from close relatives by the significant difference of components of fatty acids. The major polar lipids found in strain XK5^T were phosphatidylethanolamine, phosphatidylglycerol and two unknown phospholipids (PL1, PL2) (Fig. S2); small amounts of other unknown lipids (L1, L2, L3, AL1, AL2) were also detected. The major respiratory lipoquinone of strain XK5^T was Q-8, which is compatible with the closest relatives, the genus *Thioalkalivibrio* (Table 1).

The DNA G+C content was determined by HPLC (Mesbah *et al.*, 1989). The DNA G+C content of strain XK5^T was 59.3 mol%, which is similar to the related genera.

The results of both physiological and biochemical tests mentioned above suggest that strain XK5^T represents a novel taxon. This conclusion is also supported by the comparison of major fatty acids. All treeing methods support that the novel isolate represents a novel family within the *Chromatiales*, and the *Ectothiorhodospiraceae* is the closest relative of the novel strain based on both sequence similarity and phylogenetic tree. Differences between XK5^T and related members of the family *Ectothiorhodospiraceae* are significant (Table 1). Considering the results of phenotypic, molecular phylogenetic and chemotaxonomic analyses, we suggest that strain XK5^T represents a novel species of a new genus, for which the name *Woeseia oceani* gen. nov., sp. nov. is proposed. A novel family, *Woeseiaceae* fam. nov., is also proposed to accommodate the novel genus and species.

Description of *Woeseia* gen. nov.

Woeseia (Woe.se'i.a. N.L. fem. n. *Woeseia* named after Carl R. Woese, an American microbiologist and biophysicist, in honour of his great contributions to taxonomy and microbiology).

Cells are Gram-stain-negative, rods or bent rods, non-motile and facultatively anaerobic. Catalase is weakly positive but oxidase is negative. Does not grow without salt.

The major fatty acids are iso- $C_{17:1\omega9c}$ and iso- $C_{15:0}$. The polar lipid profile mainly consists of phosphatidylethanolamine, phosphatidylglycerol and two unknown phospholipids. Ubiquinone Q-8 is the major respiratory lipoquinone.

The type species is *Woeseia oceani*.

Description of *Woeseia oceani* sp. nov.

Woeseia oceani (o.ce.a'ni. L. gen. n. *oceani* of the ocean).

Displays the following properties in addition to those listed for the genus. Cells are 0.3–0.5 µm in diameter and 0.8–2.5 µm long. No flagella are found around the cells. Colonies are circular, viscid, brownish and 1 mm in diameter, with entire and transparent edges after incubation for 2 days at 33 °C. Growth occurs at 8–42 °C (optimum 28–35 °C) and pH 6.0–9.0 (optimum pH 7.0–8.0) with 0.5–8 % (w/v) NaCl (optimum 1–3 %). Does not hydrolyse starch, Tween-80, alginate or cellulose. According to the API ZYM kits, cells were positive for alkaline phosphatase, esterase lipase (C4), leucine arylamidase, valine arylamidase, trypsin and chymotrypsin, weakly positive for esterase lipase (C8), lipase (C14), cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. In API 20E tests, gelatin hydrolase is positive occasionally, while arginine dihydrolase, citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase are negative, and indole, acetoin (Voges–Proskauer reaction) and H₂S are not produced. According to Biolog GEN III MicroPlate assays, D-fucose, L-fucose, L-rhamnose, glycerol, D-fructose 6-phosphate, D-aspartic acid, D-glucuronic acid, glucuronamide and acetoacetic acid are oxidized. Acids can be produced from D-ribose, D-tagatose and potassium 5-ketogluconate according to API 50CH test results. Cells are susceptible to rifampicin, chloramphenicol, streptomycin, erythromycin and cefotaxime, and resistant to penicillin, lincomycin, clindamycin and trimethoprim.

The type strain, XK5^T (=ATCC BAA-2615^T=CICC 10905^T) was isolated from marine sediment from Xiaoshi Island, Weihai, China. The DNA G+C content of the type strain is 59.3 mol%.

Description of *Woeseiaceae* fam. nov.

Woeseiaceae (Woe.se.i.a.ce'ae. N.L. fem. n. *Woeseia* type genus of the family; L. suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Woeseiaceae* the family of the genus *Woeseia*).

The family is defined by phylogenetic analysis based on 16S rRNA gene sequences obtained from a sole cultured strain and some environment clones, which come from sediment or water of ocean, river, wetland, etc.

The type genus is *Woeseia*.

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