Kangiella koreensis gen. nov., sp. nov. and *Kangiella aquimarina* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea

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Two Gram-negative, non-motile, non-spore-forming, rod-shaped organisms, strains SW-125^T and SW-154^T, were isolated from tidal flat sediment of the Yellow Sea in Korea, and subjected to a polyphasic taxonomic study. Strains SW-125^T and SW-154^T grew optimally at 30–37 °C and in the presence of 2–3 % (w/v) NaCl. They contained ubiquinone-8 (Q-8) as the predominant respiratory lipoquinone and iso-C_{15:0} as the major fatty acid. The DNA G + C contents of strains SW-125^T and SW-154^T were 44 mol%. Phylogenetic trees based on 16S rRNA gene sequences revealed that the two strains form deep evolutionary lineages of descent within the γ -*Proteobacteria*. Strains SW-125^T and SW-154^T exhibited 16S rRNA gene sequence similarity levels of less than 90 % to members of the γ -*Proteobacteria* used in this analysis. Strains SW-125^T and SW-154^T showed a 16S rRNA gene sequence similarity level of 98.5 % and a mean DNA–DNA relatedness level of 9.4 %. Therefore, on the basis of phenotypic, phylogenetic and genomic data, a new genus, *Kangiella* gen. nov., is proposed to accommodate the novel strains, comprising two novel species, *Kangiella koreensis* sp. nov. (type strain, SW-125^T = KCTC 12182^T = DSM 16069^T) and *Kangiella aquimarina* sp. nov. (type strain, SW-154^T = KCTC 12183^T = DSM 16071^T).

INTRODUCTION

Tidal flats are known to be a good environment from which to isolate novel and useful micro-organisms (Yi *et al.*, 2003; Yoon *et al.*, 2003c). Some new genera and species have recently been isolated from tidal flats in Korea (Yi *et al.*, 2003; Yoon *et al.*, 2003c, f). In the course of screening novel micro-organisms from a tidal flat of the Yellow Sea in Korea, many moderately halophilic or halotolerant bacteria have been isolated and characterized taxonomically. Among these isolates, two bacterial strains, SW-125^T and SW-154^T, were investigated in detail here because they were found to form deep branches within the class *Proteobacteria* in phylogenetic analyses based on 16S rRNA gene sequences. Accordingly, the aim of this work was to determine the exact taxonomic positions of strains SW-125^T and SW-154^T using a polyphasic characterization that included phenotypic properties, detailed phylogenetic analyses based on 16S rRNA gene sequences and genomic relatedness. On the basis of the data presented, it is proposed that strains SW-125^T and SW-154^T should be placed in a new genus, *Kangiella* gen. nov., in two distinct novel species, *Kangiella*

koreensis sp. nov. and *Kangiella aquimarina* sp. nov., respectively.

METHODS

Bacterial strains and cultural conditions. Tidal flat sediment was collected from Daepo Beach, Yellow Sea, Korea, and used as source for isolation of bacterial strains. Strains SW-125^T and SW-154^T were isolated by the dilution plating technique on marine agar 2216 (MA) (Difco) at 25 °C. Cell mass of SW-125^T and SW-154^T for respiratory lipoquinone analysis and for DNA extraction was obtained from cultures in marine broth 2216 (MB) (Difco) at 30 °C. For fatty acid methyl ester (FAME) analysis, cell mass of strains SW-125^T and SW-154^T was obtained from agar plates after incubation for 7 days at 30 °C on MA.

Phenotypic characterization. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). Flagellum type was examined using TEM with cells from exponentially growing cultures. Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth at various NaCl concentrations (1–15 %) was investigated in MB or trypticase soy broth (Difco). Growth in the absence of NaCl was investigated in trypticase soy broth to which NaCl was not added. Growth at various temperatures (4–50 °C) was determined after incubation for at least 15 days on MA. Optimal pH and pH range for growth were determined in MB with pH adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber using MA and MA supplemented with

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SW-125^T and SW-154^T are AY520560 and AY520561, respectively.

nitrate that had been prepared anaerobically. Catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolysis of casein, starch, Tween 20, Tween 40, Tween 60 and Tween 80 was determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and gelatin and nitrate reduction were tested as described previously (Lanyi, 1987) with a modification that artificial sea water was used. The artificial sea water contained (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Levring, 1946). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA plates using the substrate concentrations given by Cowan & Steel (1965). H_2S production was tested as described by Bruns *et al.* (2001). Acid production from carbohydrates was determined as described by Leifson (1963). Enzyme activity was determined using the API ZYM system (bioMérieux). Requirements of peptone, yeast extract, Hutner's mineral solution (Cohen-Bazire *et al.*, 1957) and vitamin solution (Staley, 1968) for growth were determined in the liquid marine salts basal media (Baumann & Baumann, 1981) containing 0.1% each of glucose, sucrose and acetate at the following concentrations: peptone (0.05%), yeast extract (0.01%), mineral solution (2%, v/v) and vitamin solution (1%, v/v). Susceptibility to antibiotics was detected on agar plates using antibiotic discs with the following concentrations: polymyxin B (50 U), streptomycin (50 µg), penicillin (20 U), chloramphenicol (50 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), erythromycin (15 µg) and tetracycline (30 µg).

Chemotaxonomic characterization. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), except that ribonuclease T1 was used together with ribonuclease A to minimize the contamination of RNA. Respiratory lipoquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For quantitative analysis of cellular fatty acid composition, a loop of cell mass was harvested and FAMES were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

DNA–DNA hybridization. This was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values for each sample were excluded; DNA–DNA relatedness values are the mean of the remaining three values.

16S rRNA gene sequencing and phylogenetic analyses. The 16S rRNA gene was amplified by PCR using two universal primers as described by Yoon *et al.* (1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rRNA gene PCR product was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's recommendations. The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. Alignment of sequences was carried out using CLUSTAL W software (Thompson *et al.*, 1994), and the resulting alignment was then modified to remove regions containing ambiguous bases and gaps. Phylogenetic trees were inferred by using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods contained in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Jukes & Cantor (1969) with the program DNADIST. The stability of relationships was

assessed by a bootstrap analysis, based on 1000 resamplings of the neighbour-joining dataset, using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

RESULTS AND DISCUSSION

Phenotypic characteristics

Strains SW-125^T and SW-154^T grew optimally at 30–37 °C. Strain SW-125^T grew at 4 °C; strain SW-154^T did not grow at 4 °C but did grow at 10 °C. Maximum growth temperatures of strains SW-125^T and SW-154^T were 43 and 48 °C, respectively. Both strains required peptone for growth. Acid phosphatase is present in strain SW-154^T, but not in strain SW-125^T. Both strains contained ubiquinone-8 (Q-8) as the predominant respiratory lipoquinone at peak ratios of approximately 84–88%. Strains SW-125^T and SW-154^T had cellular fatty acid profiles containing large amounts of branched fatty acids, particularly iso-branched fatty acids (Table 1). The major fatty acid detected in the two strains was iso-C_{15:0} (Table 1). The DNA G+C contents of strains SW-125^T and SW-154^T were both 44 mol%. Other phenotypic properties of the two strains are given in the species descriptions below or are shown in Table 2.

Phylogeny and DNA–DNA relatedness

The almost-complete 16S rRNA gene sequences of strains SW-125^T and SW-154^T determined in this study comprised 1494 and 1496 nucleotides, respectively, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. The level of 16S rRNA gene sequence similarity

Table 1. Percentage cellular fatty acid compositions of strains SW-125^T and SW-154^T

Fatty acids representing less than 0.5% in all strains were omitted.

Fatty acid	SW-125 ^T	SW-154 ^T
C _{16:0}	1.1	1.6
iso-C _{11:0}	5.6	6.5
iso-C _{13:0}	0.4	0.6
iso-C _{15:0}	57.6	50.7
iso-C _{16:0}	0.7	1.0
iso-C _{17:0}	7.2	11.0
iso-C _{15:1} F*	1.2	1.1
iso-C _{17:1} ω _{9c}	8.6	7.0
iso-C _{11:0} 3 OH	10.5	11.7
iso-C _{15:0} 3 OH	0.9	0.7
iso-C _{17:0} 3 OH	1.0	0.6
Summed feature 1†	3.2	4.0

*The double bond positions indicated by upper-case letters are unknown.

†Summed feature 1 represents iso-C_{15:1} and/or C_{13:0} 3 OH, which could not be separated by GLC with the MIDI system.

Table 2. Differential phenotypic properties of the genus *Kangiella* and some other related genera

Taxa: 1, *Kangiella*; 2, *Alcanivorax*, data from Yakimov *et al.* (1998), Bruns & Berthe-Corti (1999) and Fernández-Martínez *et al.* (2003); 3, *Marinobacter*, data from Yoon *et al.* (2003b), Martín *et al.* (2003), Gorshkova *et al.* (2003) and Shieh *et al.* (2003); 4, *Microbulbifer*, data from González *et al.* (1997) and Yoon *et al.* (2003a, d, 2004); 5, *Marinobacterium*, data from Baumann *et al.* (1983), Bowditch *et al.* (1984), González *et al.* (1997) and Satomi *et al.* (2002); 6, *Oceanobacter*, data from Bowditch *et al.* (1984), Satomi *et al.* (2002) and Labrenz *et al.* (2003). All genera are positive for catalase and oxidase. +, Positive reaction; –, negative reaction; v, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5	6
Cell shape	Rods	Rods	Rods	Rods or cocci	Rods	Rods
Motility	–	v	+	v	+	+
Nitrate reduction	+*	v	v	– or ND	v	–
Anaerobic growth	+	v	–	–	–	–
Hydrolysis of:						
Aesculin	–	–†	– or ND	v	ND	ND
Gelatin	+	–†	v	+	–	–
Starch	–	–†	v	+	–	–
Tween 80	+	+†	+ or ND	+	+‡	+
Growth at 45 °C	v	–	v	v	–	–
Growth at 4 °C	v	v	v	–	v	–
Growth at 10 % NaCl	+	+	+	v	+‡	ND
Optimum growth temp. (°C)	30–37	25–30	20–45	25–37	37‡	20–30
Predominant ubiquinone	Q-8	ND	Q-9	Q-8	Q-8	Q-8
Major fatty acid(s)	iso-C _{15:0}	C _{16:0} , C _{18:1} ω7c, C _{16:1} ω7c	C _{18:1} ω9c, C _{16:0}	iso-C _{15:0} , iso-C _{17:1} ω9c, iso-C _{17:0}	C _{16:0} ‡	C _{16:1} , C _{18:1}
DNA G + C content (mol%)	44	53–66	53–64	58–60	55–60	54–56

*Positive under anaerobic conditions.

†Data for *Alcanivorax borkumensis*.

‡Data for *Marinobacterium georgiense*.

between strains SW-125^T and SW-154^T was 98.5 %. Phylogenetic trees based on 16S rRNA gene sequences revealed that the two strains form independent phylogenetic lineages within the evolutionary radiation enclosed by members of the *γ-Proteobacteria* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strains SW-125^T and SW-154^T formed a cluster supported by a bootstrap resampling value of 100 %, and joined the phylogenetic clade comprising authentic pseudomonads and several genera, *Marinobacterium*, *Marinomonas*, *Oceanospirillum*, *Pseudospirillum*, *Shewanella*, *Alteromonas*, *Oceanobacter*, *Halomonas*, *Marinospirillum*, *Microbulbifer*, *Marinobacter* and *Alcanivorax*, by a bootstrap resampling value of 61.1 % (Fig. 1). Strains SW-125^T and SW-154^T exhibited 16S rRNA gene sequence similarity levels of less than 89.3 % (*Microbulbifer hydrolyticus* ATCC 700072^T) and 89.0 % (*Microbulbifer hydrolyticus* ATCC 700072^T and *Oceanobacter kriegii* IFO 15467^T), respectively, to species used in the phylogenetic analysis (Fig. 1). Strains SW-125^T and SW-154^T exhibited a mean level of DNA–DNA relatedness of 9.4 %, when their DNA was used individually as a labelled DNA probe, indicating that the two strains represent members of different genomic species (Wayne *et al.*, 1987).

Conclusion

The phylogenetic analyses based on 16S rRNA gene sequences indicated that strains SW-125^T and SW-154^T did not fall within the radiation encompassed by a recognized genus but form distinct lineages within the *γ-Proteobacteria*. The 16S rRNA gene sequence similarity values to members of the *γ-Proteobacteria* were very low (less than 90 %). Strains SW-125^T and SW-154^T are differentiated from some phylogenetically related genera by several phenotypic characteristics, particularly respiratory lipoquinone and fatty acid profiles (Table 2). The genera *Pseudomonas*, *Halomonas* and *Marinobacter* have been observed to have ubiquinone-9 as the predominant respiratory lipoquinone (Oyaizu & Komagata, 1983; Dobson & Franzmann, 1996; Yumoto *et al.*, 2001; Yoon *et al.*, 2003b). *Shewanella* species simultaneously contain both menaquinones and ubiquinones (Venkateswaran *et al.*, 1999; Bozal *et al.*, 2002). The fatty acid patterns, particularly the major fatty acid(s), distinguish strains SW-125^T and SW-154^T from some genera, including *Alcanivorax*, *Alteromonas*, *Microbulbifer*, *Marinobacterium*, *Marinomonas*, *Methylococcus*, *Methylobacterium*, *Oceanospirillum* and *Oceanobacter* (Bowman *et al.*, 1993; González *et al.*, 1997; Fernández-Martínez *et al.*, 2003; Satomi *et al.*,

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic positions of strains SW-125^T and SW-154^T within the radiation of representatives of the γ -Proteobacteria. Bar, 0.01 substitution per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branch points. Dots indicate that the corresponding nodes are also recovered in the maximum-likelihood tree.

2002; Labrenz *et al.*, 2003; Romanenko *et al.*, 2003; Yoon *et al.*, 2003a, e). Therefore, it is appropriate that strains SW-125^T and SW-154^T are allocated in a new genus within the γ -Proteobacteria. Although strains SW-125^T and SW-154^T are similar in most phenotypic properties, they are considered to belong to different species on the basis of DNA–DNA relatedness and differences in their phenotypic properties, such as temperature for growth. On the basis of the phenotypic and phylogenetic data and the genomic distinctiveness, strains SW-125^T and SW-154^T should be

placed in a new genus, *Kangiella* gen. nov., in two novel species, *Kangiella koreensis* sp. nov. and *Kangiella aquimarina* sp. nov., respectively.

Description of *Kangiella* gen. nov.

Kangiella (Kan.gi.el'la. N.L. dim. fem. n. *Kangiella* named to honour Professor Kook Hee Kang, a Korean microbiologist, for his contribution to microbial research).

Gram-negative, non-motile, non-spore-forming rods.

Colonies are smooth, raised, circular to slightly irregular and light brown in colour on MA. Catalase- and oxidase-positive. Urease-negative. Nitrate reduction is negative under aerobic conditions. Nitrate is reduced to nitrogen gas under anaerobic conditions. NaCl and peptone are required for growth. The predominant respiratory lipoquinone is ubiquinone-8 (Q-8). The major fatty acid is iso-C_{15:0}. The DNA G + C content is 44 mol% (as determined by HPLC).

The type species is *Kangiella koreensis*.

Description of *Kangiella koreensis* sp. nov.

Kangiella koreensis (ko.re.en'sis. N.L. fem. adj. *koreensis* pertaining to Korea, from where the organism was isolated).

Cells are rods, 0.2–0.5 × 1.5–4.5 µm. Non-motile. Non-spore-forming. Gram-negative. Colonies are smooth, raised, circular to irregular, light yellowish-brown in colour and 2.0–3.0 mm in diameter after 7 days incubation at 30 °C on MA. Optimal growth temperature is 30–37 °C. Growth occurs at 4 °C. Maximum growth temperature is 43 °C. Optimal growth pH is 7.0–8.0. Growth occurs at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 2–3 % NaCl. Growth occurs in the presence of 12 % NaCl, but not without NaCl or in the presence of more than 13 % NaCl. Growth under anaerobic conditions occurs on MA supplemented with nitrate. Casein, tyrosine, Tween 20, Tween 40 and Tween 60 are hydrolysed. Hypoxanthine and xanthine are not hydrolysed. H₂S is not produced. Nitrate is not reduced under aerobic conditions. Nitrate is reduced to nitrogen gas under anaerobic conditions. Acid is not produced from the following sugars: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, *myo*-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-trehalose or D-xylose. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), cystine arylamidase, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to polymyxin (50 U), streptomycin (50 µg), penicillin (20 U), chloramphenicol (50 µg), ampicillin (10 µg), cephalothin (30 µg) and erythromycin (15 µg). Resistant to novobiocin (5 µg) and tetracycline (30 µg). The predominant respiratory lipoquinone is ubiquinone-8 (Q-8). The major fatty acid is iso-C_{15:0}. The DNA G + C content is 44 mol% (as determined by HPLC).

The type strain (SW-125^T = KCTC 12182^T = DSM 16069^T) was isolated from tidal flat sediment of the Yellow Sea, Korea.

Description of *Kangiella aquimarina* sp. nov.

Kangiella aquimarina (a.qui.ma.ri'na. L. n. *aqua* water; L. adj. *marinus* of the sea; N.L. fem. adj. *aquimarina* pertaining to sea water).

Cells are rods, 0.2–0.5 × 1.5–4.5 µm. Non-motile. Non-spore-forming. Gram-negative. Colonies are smooth, raised, circular to irregular, light yellowish-brown in colour and 2.0–3.0 mm in diameter after 7 days incubation at 30 °C on MA. Optimal growth temperature is 30–37 °C. Growth occurs at 10 °C, but not at 4 °C. Maximum growth temperature is 48 °C. Optimal growth pH is 7.0–8.0. Growth occurs at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 2–3 % NaCl. Growth occurs in the presence of 12 % NaCl, but not without NaCl or in the presence of more than 13 % NaCl. Growth under anaerobic conditions occurs on MA supplemented with nitrate. Casein, tyrosine, Tween 20, Tween 40 and Tween 60 are hydrolysed. Hypoxanthine and xanthine are not hydrolysed. H₂S is not produced. Nitrate is not reduced under aerobic conditions. Nitrate is reduced to nitrogen gas under anaerobic conditions. Acid is not produced from the following sugars: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, *myo*-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-trehalose or D-xylose. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to polymyxin (50 U), streptomycin (50 µg), penicillin (20 U), chloramphenicol (50 µg), ampicillin (10 µg), cephalothin (30 µg) and erythromycin (15 µg). Resistant to novobiocin (5 µg) and tetracycline (30 µg). The predominant respiratory lipoquinone is ubiquinone-8 (Q-8). The major fatty acid is iso-C_{15:0}. The DNA G + C content is 44 mol% (as determined by HPLC).

The type strain (SW-154^T = KCTC 12183^T = DSM 16071^T) was isolated from tidal flat sediment of the Yellow Sea, Korea.

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