Reinekea aestuarii sp. nov., isolated from tidal flat sediment

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Two Gram-negative, non-motile, non-pigmented and curved rod-shaped bacterial strains, designated IMCC4489^T and IMCC4451, were isolated from a tidal flat sediment of the Yellow Sea. Strains IMCC4489^T and IMCC4451 shared 99.9 % 16S rRNA gene sequence similarity and 78.5 % DNA–DNA relatedness, which suggested that they belonged to the same species. The isolates were most closely related to *Reinekea blandensis* MED297^T (98.7–98.8 % 16S rRNA gene sequence similarity) and *Reinekea marinisedimentorum* DSM 15388^T (95.3–95.4 %). DNA–DNA relatedness between the strains and *R. blandensis* CCUG 52066^T was 31–34 %. Strains IMCC4489^T and IMCC4451 could also be differentiated from the type strains of the two recognized *Reinekea* species by several phenotypic properties. The DNA G+C content was 51.3–51.5 mol% and the major isoprenoid quinone was Q-8. On the basis of the data obtained in this study, it is proposed that strains IMCC4489^T and IMCC4451 represent a novel species, *Reinekea aestuarii* sp. nov. The type strain is IMCC4489^T (=KCTC 22813^T =KCCM 42938^T =NBRC 106079^T).

The genus *Reinekea* (Romanenko *et al.*, 2004) in the order *Oceanospirillales* (Garrity *et al.*, 2005) accommodates Gram-negative, chemoheterotrophic, aerobic or facultatively anaerobic, non-pigmented, motile, rod-shaped bacteria. At present, the genus *Reinekea* comprises two recognized species, *Reinekea marinisedimentorum* (Romanenko *et al.*, 2004) and *Reinekea blandensis* (Pinhassi *et al.*, 2007). *R. marinisedimentorum* is the type species of the genus, isolated from a coastal sediment sample, and *R. blandensis* MED297^T is a genome-sequenced bacterium, isolated from a seawater sample. Both species require sodium ions for growth. During a survey of microbial diversity inhabiting tidal flat sediments of the Yellow Sea, two bacterial strains, IMCC4489^T and IMCC4451, were isolated using a dilution-plating method and subjected to a taxonomic study. On the basis of the present taxonomic study, we propose the inclusion of these two strains in a novel species of the genus *Reinekea*.

Strains IMCC4489^T and IMCC4451 were isolated from a tidal flat sediment sample collected off the Yellow Sea coast (Kanghwa tidal flat, Korea; 37° 36′ 07″ N 126° 29′ 10″ E; 20 °C). The sediment sample (1 g wet weight) was homogenized with a glass mixer in 100 ml sterile seawater. A 100 µl sample of the homogenate was spread onto an oligotrophic medium, 1/10 R2A agar [R2A agar (BD Difco) diluted 1 : 10 (v/v) with aged seawater] and the agar plates were incubated aerobically at 20 °C for 30 days. The non-pigmented colonies of the isolates were subcultivated on marine agar 2216 (MA; BD Difco) for 7 days. After the optimum temperature for growth had been determined, the strains were routinely grown on MA at 25 °C. For phenotypic, chemotaxonomic and genotypic comparisons between the isolates and the genus *Reinekea*, two reference type strains, *R. marinisedimentorum* DSM 15388^T and *R. blandensis* CCUG 52066^T, were obtained from the DSMZ and the CCUG, respectively.

Genomic DNA was extracted from purified colonies using a DNeasy tissue kit (Qiagen), according to the manufacturer's instructions. DNA–DNA relatedness among the isolates and reference strains was determined by a fluorometric method (Ezaki *et al.*, 1989) using photobiotin-labelled DNA probes and microdilution wells. The 16S rRNA gene was amplified using the primers 27F-B and 1492R and sequenced as described by Cho & Giovannoni (2004). The resultant almost-complete 16S rRNA gene sequences of strains IMCC4489^T and IMCC4451 (both 1466 bp) were imported into the ARB software package (Ludwig *et al.*, 2004) and aligned on the basis of the secondary structure of the 16S rRNA. Sequence similarities were determined in the ARB package and also confirmed by the EzTaxon server (Chun *et al.*, 2007). Phylogenetic trees were generated using the neighbour-joining method (Saitou & Nei, 1987) with Jukes–Cantor distances (Jukes & Cantor, 1969) and the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IMCC4489^T and IMCC4451 are GQ456131 and GQ280347, respectively.

A thin-layer chromatogram of the polar lipids of strain IMCC4489^T is available as supplementary material with the online version of this paper.

Strains IMCC4489^T and IMCC4451 shared 99.9 % 16S rRNA gene sequence similarity and exhibited 78.5 % DNA–DNA relatedness, and thus they were considered to be members of the same genomic species (Wayne *et al.*, 1987). On the basis of 16S rRNA gene sequence comparisons, the isolates were most closely related to *R. blandensis* MED297^T (98.7–98.8 % 16S rRNA gene sequence similarity), *R. marinisedimentorum* DSM 15388^T (95.3–95.4 %) and *Saccharospirillum impatiens* EL-105^T (93.7–93.8 %). In all of the phylogenetic trees, strains IMCC4489^T and IMCC4451, *R. marinisedimentorum* DSM 15388^T and *R. blandensis* MED297^T formed a well-supported clade within the order *Oceanospirillales* (Fig. 1), indicating that strains IMCC4489^T and IMCC4451 were members of the genus *Reinekea*. DNA–DNA relatedness between the isolates and *R. blandensis* CCUG 52066^T was 31–34 %. The values of <97 % 16S rRNA gene sequence similarity between the isolates and *R. marinisedimentorum* DSM 15388^T and <70 % DNA–DNA relatedness between the isolates and *R. blandensis* CCUG 52066^T indicated that strains IMCC4489^T and IMCC4451 represented a genomic species separate from the two recognized *Reinekea* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006).

For phenotypic characterization, strains IMCC4489^T and IMCC4451 were grown on MA at 25 °C unless otherwise stated. Cellular morphology and cell size were examined by both transmission electron microscopy (CM200; Philips) and phase-contrast microscopy (80i; Nikon). Flagellum-based motility was determined using a wet mount made from fresh cultures grown in marine broth at 25 °C for 24 h and gliding motility was determined using the hanging-drop method. Growth of the strains under anaerobic conditions was tested in an Anaero Pack anaerobic chamber (Mitsubishi Gas Chemical) for 3 weeks. Conditions for growth were determined at 4, 10, 15, 20, 25, 30, 37 and 42 °C, at pH 4.0–12.0 (at intervals of 0.5 pH units) and with 0–15 % (w/v) NaCl in NaCl-free artificial seawater medium (ASW; Choo *et al.*, 2007) amended with 5.0 g peptone and 1.0 g yeast extract l^{-1}. Catalase activity was determined with the addition of 3.0 % hydrogen peroxide to fresh colonies and oxidase activity was determined using Kovács' solution. Hydrolysis of gelatin, casein, starch, DNA, chitin and Tween 80, production of H_2S and the Voges–Proskauer test were determined according to the methods described by Smibert & Krieg (1994) except for using MA as a basal medium. Other biochemical tests and carbon source oxidation tests were carried out for the isolates and reference strains using API 20NE, API ZYM and API 50CH test strips (bioMérieux) and GN2 MicroPlates (Biolog) according to the manufacturers' instructions except for suspending the cells in ASW medium. The API 50CH results were recorded after 3 days.

The phenotypic characteristics of strains IMCC4489^T and IMCC4451 are given in the species description and Table 1. Briefly, cells of the isolates were Gram-negative (by Gram staining and KOH test), chemoheterotrophic, facultatively anaerobic, oxidase- and catalase-positive, non-motile, non-pigmented, curved rods that required NaCl for growth. Strains IMCC4489^T and IMCC4451 exhibited slightly different patterns of acid production from carbohydrates and carbon source oxidation. A number of phenotypic characteristics differentiated the isolates from one or both of the reference strains, including anaerobic growth, motility, nitrate reduction, enzyme activities and acid production and carbon source oxidation patterns (Table 1).

The DNA G+C content was determined using the HPLC method (Mesbah *et al.*, 1989) with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco). The fatty acid methyl esters of the isolates and the reference strains were extracted from fresh cultures grown on MA at 25 °C for 5 days and determined by the Sherlock Microbial Identification System (MIDI), according to the manufacturer's instructions. The polar lipids of strain IMCC4489^T were determined using two-dimensional TLC on silica gel according to Minnikin *et al.* (1984). Isoprenoid quinones

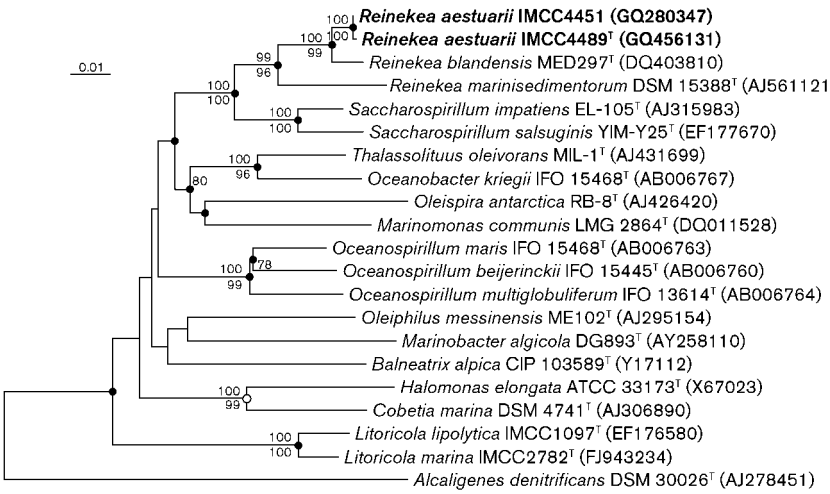

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains IMCC4489^T and IMCC4451 and their relatives in the order *Oceanospirillales*. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. The open circle indicates that the corresponding node was also recovered in trees generated with the maximum-parsimony method. Bootstrap values (>70 %) are shown at branch nodes for the neighbour-joining and maximum-parsimony analysis (above and below nodes, respectively). *Alcaligenes denitrificans* JCM 5490^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics that differentiate strains IMCC4489^T and IMCC4451 from the type strains of *Reinekea* species

Strains: 1, *Reinekea aestuarii* sp. nov. IMCC4489^T and IMCC4451 (for variable characters, result given for IMCC4489^T/IMCC4451); 2, *R. blandensis* CCUG 52066^T; 3, *R. marinisedimentorum* DSM 15388^T. Data were obtained in this study unless indicated. All strains were positive for aesculin hydrolysis, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid production from aesculin ferric citrate and oxidation of cellobiose, D-fructose, α-D-glucose, maltose, D-mannitol, D-sorbitol, sucrose, trehalose and D-glucose 6-phosphate. All strains were negative for indole production, arginine dihydrolase, urease, α-chymotrypsin, α-fucosidase, β-glucuronidase, α-mannosidase, acid production from D-arabinose, D- and L-fucose, D-lyxose, melezitose, L-rhamnose, D-ribose, L-sorbose, D-tagatose, D- and L-xylose, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, D-adonitol, D- and L-arabitol, dulcitol, erythritol, inositol, D-sorbitol, xylitol, inulin, potassium gluconate and potassium 2-ketogluconate and oxidation of Tweens 40 and 80, L-arabinose, L-fucose, L-rhamnose, *myo*-inositol, xylitol, pyruvic acid methyl ester, succinic acid monomethyl ester, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alanyl glycine, glycyl L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D- and L-serine, L-threonine, DL-carnitine, urocanic acid, phenylethylamine, putrescine or 2,3-butanediol. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3
Temperature range for growth (°C)	15–42	15–42[a*]	4–37[b]
Growth with 12 % NaCl	−	+[a]	−[b]
Anaerobic growth	+	−[a]	+[b]
Motility	−	+[a]	+[b]
H_2S production	−	+	+
Hydrolysis of:			
Chitin, Tween 80	+	+	−
Gelatin	+	−	−
Starch, casein, DNA	−	+	−
API 20NE results			
Gelatin liquefaction	W	+	−
Nitrate reduction, glucose fermentation, β-galactosidase	−	−	+
Enzyme activities (API ZYM)			
Valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase	+	+	−
Trypsin	+	−	−
Lipase (C14), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase	−	−	+
Acid production (API 50CH) from:			
L-Arabinose, arbutin, glycogen	+	+	−
Cellobiose	+/−	+	+
N-Acetylglucosamine	+	−	−
Methyl α-D-glucopyranoside, potassium 5-ketogluconate, lactose	+/−	−	−
Glycerol, D-galactose, gentiobiose, maltose, trehalose	+/−	−	+
D-Glucose, D-fructose, D-mannose, raffinose	−	−	+
Starch	−	+	−
Carbon source oxidation (Biolog GN2)			
α-Cyclodextrin, dextrin, glycogen, *N*-acetyl-D-glucosamine, D-galactose, gentiobiose, lactose, lactulose, D-mannose, melibiose, raffinose, turanose, D-galacturonic acid, DL-lactic acid, succinic acid, L-alanine, L-glutamic acid, glycyl L-glutamic acid, inosine, uridine, thymidine, DL-α-glycerol phosphate	+	+	−
i-Erythritol, L-alaninamide	+/−	+	−
L-Asparagine, L-aspartic acid	+	−	−
α-Ketoglutaric acid	+/−	−	−
α-Hydroxybutyric acid	−	+	+
D-Psicose	−	−	+
D-Arabitol, β-hydroxybutyric acid, L-ornithine	−	+	−
Methyl β-D-glucoside, D-glucosaminic acid, D-alanine, α-D-glucose 1-phosphate	−/+	−	−
Major polar lipids†	PG, DPG, PE, PS	PG, DPG, PE, PI[a]	PG, DPG, PE, PI[b]
DNA G+C content (mol%)	51.3/51.5	52.4[a]	51.1[b]

*Data taken from: *a*, Pinhassi *et al.* (2007); *b*, Romanenko *et al.* (2004).

†DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

were extracted by TLC according to Minnikin *et al.* (1984) and analysed by using HPLC (Collins, 1985).

The DNA G+C contents of strains IMCC4489^T and IMCC4451 were 51.3 and 51.5 mol%, respectively, only 0.2–1.1 mol% different from those of *R. blandensis* CCUG 52066^T and *R. marinisedimentorum* DSM 15388^T (Table 1). The major cellular fatty acid components of strain IMCC4489^T were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 27.4 %), iso-$C_{16:0}$ (16.7 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 10.7 %), $C_{16:0}$ (9.6 %), $C_{17:1}\omega8c$ (6.8 %) and iso-$C_{18:0}$ (5.5 %). The fatty acid profiles of strains IMCC4489^T and IMCC4451 were largely similar to that of *R. blandensis* CCUG 52066^T except for the proportions of $C_{17:1}\omega6c$ and iso-$C_{16:0}$, but they differed from that of *R. marinisedimentorum* DSM 15388^T in the presence of iso-$C_{16:0}$ and iso-$C_{18:0}$ and in the proportions of $C_{17:0}$ and $C_{17:1}\omega6c$ (Table 2). The polar lipids of strain IMCC4489^T included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, two unidentified phospholipids and two unidentified polar lipids (Supplementary Fig. S1, available in IJSEM Online). The respiratory quinones detected in strains IMCC4489^T and IMCC4451 were ubiquinones, with Q-8 predominating and Q-9 present in minor amounts, as described for the genus *Reinekea*.

Table 2. Cellular fatty acid compositions of strains IMCC4489^T and IMCC4451 and type strains of the genus *Reinekea*

Strains: 1, *R. aestuarii* sp. nov. IMCC4489^T; 2, *R. aestuarii* sp. nov. IMCC4451; 3, *R. blandensis* CCUG 52066^T; 4, *R. marinisedimentorum* DSM 15388^T. Values are percentages of total fatty acids and were obtained in this study. Fatty acids that represented <1.0 % in all strains are not shown. tr, Trace (<1.0 %); −, not detected.

Fatty acid	1	2	3	4
$C_{14:0}$	tr	tr	tr	1.2
$C_{16:0}$	9.6	10.1	7.0	12.2
$C_{17:0}$	6.5	6.6	10.0	20.2
$C_{18:0}$	1.1	tr	tr	tr
$C_{15:1}\omega8c$	tr	tr	tr	1.1
$C_{15:1}\omega6c$	tr	tr	tr	1.6
$C_{17:1}\omega8c$	6.8	7.7	8.8	11.3
$C_{17:1}\omega6c$	3.4	3.9	10.9	18.9
iso-$C_{16:0}$	16.7	15.8	9.3	−
iso-$C_{18:0}$	5.5	4.4	1.9	−
iso-$C_{18:1}$ H	1.3	1.3	tr	−
$C_{10:0}$ 3-OH	1.0	tr	−	−
iso-$C_{14:0}$ 3-OH	1.2	1.2	1.3	−
11-Methyl $C_{18:1}\omega7c$	1.2	tr	tr	−
Summed features*				
1	tr	tr	tr	1.8
3	10.7	14.4	11.6	18.0
7	1.8	tr	tr	−
8	27.4	28.1	30.1	12.0

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 consisted of iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 3 consisted of $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; summed feature 7 consisted of $C_{19:1}\omega7c$ and/or $C_{19:1}\omega6c$; summed feature 8 consisted of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

The formation of a robust clade by strains IMCC4489^T and IMCC4451 and the type strains of species of the genus *Reinekea* and the chemotaxonomic characteristics supported the isolates being assigned to the genus *Reinekea*. However, the low DNA–DNA relatedness between the isolates and *R. blandensis* CCUG 52066^T, the low 16S rRNA gene sequence similarity between the isolates and *R. marinisedimentorum* DSM 15388^T and several phenotypic characteristics suggested that strains IMCC4489^T and IMCC4451 represent a novel species of the genus *Reinekea*, for which the name *Reinekea aestuarii* sp. nov. is proposed.

Description of *Reinekea aestuarii* sp. nov.

Reinekea aestuarii (a.es.tu.a′ri.i. L. gen. n. *aestuarii* of a tidal flat).

Gram-negative, chemoheterotrophic, oxidase- and catalase-positive, non-motile, non-pigmented and facultatively anaerobic. Grows anaerobically but aerobic growth is much better than anaerobic growth. Cells are curved rods (0.4–0.5 × 1.2–2.5 μm) that divide by binary fission. After growth on MA at 25 °C for 5 days, colonies are 0.2–0.4 mm in diameter, circular, convex, opaque, smooth and beige. Grows at 15–42 °C (optimum 25 °C), at pH 5.0–12.0 (optimum pH 6.0–8.0) and with 0.5–10.0 % NaCl (optimum 2.0–5.0 % NaCl). Gelatin, chitin and Tween 80 are hydrolysed, but DNA, starch and casein are not. H_2S is not produced. With API 20NE, positive for aesculin hydrolysis but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease and β-galactosidase. With API ZYM, positive for acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), cystine arylamidase, leucine arylamidase, valine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. With API 50CH, positive for acid production from L-arabinose, arbutin, glycogen and *N*-acetylglucosamine and variable for cellobiose, glycerol, D-galactose, gentiobiose, lactose, maltose, trehalose, methyl α-D-glucopyranoside and potassium-5-ketogluconate. With GN2 MicroPlates, positive for oxidation of cellobiose, α-cyclodextrin, glycogen, *N*-acetyl-D-glucosamine, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-sorbitol, sucrose, trehalose, turanose, acetic acid, D-galacturonic acid, DL-lactic acid, succinic acid, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, inosine, uridine, thymidine, glycerol, DL-α-glycerol phosphate and D-glucose 6-phosphate and variable for oxidation of i-erythritol, L-alaninamide, α-ketoglutaric acid, methyl β-D-glucoside,

D-glucosaminic acid, D-alanine and α-D-glucose 1-phosphate. The major respiratory quinone is Q-8. The polar lipids of the type strain consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, two unidentified phospholipids and two unidentified polar lipids. The predominant cellular fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), iso-$C_{16:0}$ and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The DNA G+C content of the type strain is 51.3 mol%.

The type strain is IMCC4489^T (=KCTC 22813^T =KCCM 42938^T =NBRC 106079^T), isolated from a tidal flat of the Yellow Sea, Korea.

Acknowledgements

This study was supported by the 21C Frontier Program of Microbial Genomics and Applications, by the Korea Research Foundation Grant funded by MEST (KRF-2007-314-C00265) and by a grant from the Gyeonggi Sea Grant Program funded by the MLTM of the Korean government.

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