Reichenbachiella faecimaris sp. nov., isolated from a tidal flat, and emended descriptions of the genus *Reichenbachiella* and *Reichenbachiella agariperforans*

In-Tae Cha,¹ Yong-Sik Oh,¹ Soo-Je Park,¹ Byoung-Joon Park,¹ Je-Kwan Lee,¹ Chae-Sung Lim,¹ A-Rum Park,¹ Jae-Soo Yoo,² Dong-Hun Lee,¹ Sung-Keun Rhee¹ and Dong-Hyun Roh¹

Correspondence

Dong-Hyun Roh

dhroh@chungbuk.ac.kr

¹Department of Microbiology and Biotechnology Research Institute, Chungbuk National University, Cheongju 361-763, Chungbuk, Republic of Korea

²School of Electrical and Computer Engineering, Chungbuk National University, Cheongju 361-763, Chungbuk, Republic of Korea

A taxonomic study was carried out on two bacterial strains, PCP11^T and PCP104, isolated from a tidal flat of the Yellow Sea, Korea. Comparative 16S rRNA gene sequence studies showed that these strains belonged to the family *Cytophagaceae*, phylum *Bacteroidetes*. Strains PCP11^T and PCP104 shared 99.4% sequence similarity and were related most closely to *Reichenbachiella agariperforans* KMM 3525^T (95.8 and 96.0% sequence similarity, respectively). Members of the genera *Fulvivirga*, *Roseivirga*, *Fabibacter* and *Marinoscillum* were the next closest relatives of the new isolates, with sequence similarities $\leq 91\%$. The two isolates were Gram-staining-negative, strictly aerobic, gliding bacteria. They grew in the presence of 1–5% NaCl, at pH 5.5–8.5 and at 4–35 °C. Strains PCP11^T and PCP104 shared a number of physiological and biochemical properties with *Reichenbachiella agariperforans* KMM 3525^T, but they differed from this strain in the hydrolysis of biopolymers and in the production of carotenoid and flexirubin-type pigments. Both strains possessed iso-C_{15:0}, summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{15:0} as major cellular fatty acids. The major respiratory quinone was menaquinone 7 (MK-7). The G + C contents of the genomic DNA of strains PCP11^T and PCP104 were 39.6 and 41.9 mol%, respectively. On the basis of phenotypic data and phylogenetic inference, it is proposed that the two isolates represent a novel species, *Reichenbachiella faecimaris* sp. nov., with strain PCP11^T (=KACC 14523^T =JCM 16588^T) as the type strain. Emended descriptions of the genus *Reichenbachiella* and *Reichenbachiella agariperforans* are also proposed.

The genus *Reichenbachia* and the species *Reichenbachia agariperforans* (family *Cytophagaceae*, phylum *Bacteroidetes*; Euzéby, 1997; Ludwig *et al.*, 2008) were originally proposed by Nedashkovskaya *et al.* (2003) to accommodate a Gram-staining-negative, strictly aerobic, oxidase-, catalase- and alkaline phosphatase-positive, rod-shaped bacterial strain that was motile by gliding. As the genus name *Reichenbachia* was illegitimate, it was subsequently changed to *Reichenbachiella* and new, modified genus and species descriptions were published (Nedashkovskaya *et al.*, 2005). At the time of writing, *Reichenbachiella agariperforans* remained the only species in the genus. In this study, we report on the polyphasic taxonomic characterization of the

Reichenbachiella-like bacterial strains PCP11^T and PCP104, isolated from a tidal flat of the Yellow Sea, Korea. The two strains are proposed to represent a novel species of the genus *Reichenbachiella*.

During a study of bacterial diversity at a tidal flat of the Yellow Sea in the Muchangpo region of Korea (36° 14' 28" N 126° 31' 54" E), two novel bacterial strains were isolated. For isolation of bacteria, tidal-flat sediment collected in sterile conical tubes was serially diluted with filter-sterilized (0.2 µm pore size; Millipore) natural seawater containing 0.01% yeast extract. After incubation at 25 °C under aerobic conditions for 2 weeks, an aliquot of the last dilution showing turbidity was spread onto artificial seawater (ASW) agar and incubated at 25 °C for 2 weeks. ASW broth and agar contained (l⁻¹) 1 g yeast extract, 23.6 g NaCl, 0.64 g KCl and 4.53 g MgCl₂·6H₂O, with

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PCP11^T and PCP104 are GU143096 and GU143095, respectively.

1.5 % agar for solidification. Single colonies were purified by transfer onto marine agar 2216E (MA; Difco) and incubation at 25 °C for 3 days. The strains were stored at –80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (w/v) glycerol.

Genomic DNA of the two isolates was extracted using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primers 27F (5'-AGAGT-TTGATCMTGGCTCAG-3'; *Escherichia coli* positions 8–27) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; 1492–1510) (Park *et al.*, 2006; Weisburg *et al.*, 1991). Purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Korea) using primers 27F, 338F, 786R and 1492R (Weisburg *et al.*, 1991). Nearly complete 16S rRNA gene sequences were compiled using the SeqMan software (DNASTAR) and sequences of related taxa were obtained from the GenBank database. Sequence alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Nucleotide similarity values were calculated using the EzTaxon program (<http://www.eztaxon.org>; Chun *et al.*, 2007) and evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms by using the MEGA 4 program (Tamura *et al.*, 2007). Bootstrap analysis was performed with 1000 resampled datasets using the built-in programs of MEGA 4.

On the basis of analysis of 16S rRNA gene sequences (1429 bp for both strains), strains PCP11^T and PCP104 were shown to belong to the family *Cytophagaceae*, phylum *Bacteroidetes*, and the highest sequence similarity was found with *R. agariperforans* KMM 3525^T (95.8 and 96.0 %, respectively) (Fig. 1). Members of the genera *Fulvivirga*, *Roseivirga*, *Fabibacter* and *Marinoscillum* were the next closest relatives of the new isolates, with sequence similarities ≤91 %. Strains PCP11^T and PCP104 shared 99.4 % sequence similarity; they clearly belonged to the genus *Reichenbachiella*, as shown by the high bootstrap values (both 100 %) of the neighbour-joining and maximum-parsimony phylogenetic trees (Fig. 1). Hence, *R. agariperforans* KCTC 12369^T was obtained from the Korean Collection for Type Cultures (Daejeon, Republic of Korea) and used as a reference strain in most phenotypic tests.

Strains PCP11^T and PCP104 and *R. agariperforans* KCTC 12369^T were routinely grown on MA at 28 °C for 3 days. Morphology and size of cells grown under these conditions were observed by phase-contrast microscopy (Nikon 80i) and the production of endospores was examined using cells grown at 28 °C for 14 days on MA. The presence of gliding motility was assessed by microscopic observation of a hanging drop from a fresh MB culture and of the edge of a young colony on a glass slide coated with MA (Agarwal

et al., 1997; Bernardet *et al.*, 2002). Gram staining was performed using the BD Gram stain kit according to the instructions of the manufacturer. Catalase and oxidase activities were assessed by bubble production in 3 % (v/v) H₂O₂ and using the bioMérieux Orientation test kit, respectively. Growth under anaerobic conditions was assessed after 7 days of incubation on MA at 28 °C in the GasPak EZ anaerobic container system (BD). The production of flexirubin-type pigments was assessed by using the KOH test (Reichenbach, 1989; Bernardet *et al.*, 2002). Carotenoid pigments were extracted from cell pellets washed with methanol (4 ml) at 4 °C overnight and the presence of absorption maxima in the UV–visible spectrum at 450 and 480 nm, characteristic of carotenoids in *R. agariperforans* KCTC 12369^T, was examined.

Growth was assessed at 0, 4, 10, 20, 25, 30, 35 and 40 °C after 3 days of incubation in MB. Requirement for and tolerance of NaCl were evaluated after incubation at 28 °C for 2 weeks in a liquid medium containing (l⁻¹) 1 g yeast extract, 5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g CaCl₂, 0.05 g FeSO₄·7H₂O and 0.028 g NH₄Cl and supplemented with 0–20 % (w/v) NaCl (at intervals of 1 %). Growth at pH 5.0–9.0 (at intervals of 0.5 pH units) was assessed in MB at 28 °C for 3 days. The medium was adjusted to the desired pH with 1 M HCl or NaOH. Acid production from various substrates, assimilation of carbon sources and enzyme activities were determined using API 20NE, API ID32GN, API 50CH and API ZYM galleries according to the instructions of the manufacturer (bioMérieux) except that the culture was suspended in 2 % (w/v) sea salts solution supplemented with 0.01 % yeast extract (González *et al.*, 1997) and that galleries were incubated at 28 °C for 3 days. *R. agariperforans* KCTC 12369^T was also included in the API tests and all tests were performed under the same conditions. Hydrolysis of starch (soluble or from rice; 1 %, w/v), agar (1.5 %, w/v), alginate (1 %, w/v), gelatin (1 %, w/v), urea (1 %, w/v), CM-cellulose (1 %, w/v), filter paper, chitin (1 %, w/v), casein (1 %, w/v) and Tweens 20, 40, 60 and 80 (1 %, v/v) was tested on ASW agar (Smibert & Krieg, 1994). DNA hydrolysis was assessed on DNase test agar with methyl green (Difco) supplemented with 3 % NaCl (final concentration). The production of H₂S was tested on peptone iron agar (Difco) supplemented with 3 % NaCl (final concentration). The following antibiotics were tested on MA at 28 °C for 7 days using the disc-diffusion method with laboratory-prepared discs containing the indicated amounts (µg per disc): ampicillin (10), chloramphenicol (25), erythromycin (15), gentamicin (10), kanamycin (30), mitomycin C (50), penicillin G (10), streptomycin (10) and tetracycline (30).

The phenotypic characteristics of strains PCP11^T and PCP104 are given in the species description and are compared with those of *R. agariperforans* KCTC 12369^T in Table 1.

For cellular fatty acid analysis, strains PCP11^T and PCP104 and *R. agariperforans* KCTC 12369^T were cultivated on MA

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains PCP11^T and PCP104, *R. agariperforans* KMM 3525^T and representatives of the family *Cytophagaceae*. The sequence of *Rhodothermus marinus* DSM 4252^T was used as an outgroup and GenBank accession numbers are given in parentheses. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points (neighbour-joining, above line; maximum-parsimony, below line). Bar, 0.02 substitutions per nucleotide position.

(pH 7.5) for 3 days at 28 °C. Cellular fatty acids were saponified, methylated, extracted and analysed (Sasser, 1990) according to the protocol of the Sherlock Microbial Identification System (MIDI) by using the TSBA 40 method. Extracts were analysed by GC (Hewlett Packard 6890) and identified by comparing the fatty acid profiles to the TSBA 40 database provided with the Sherlock software 2.95. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum and re-extracted in n-hexane–water (1:1, v/v). The crude menaquinones in n-hexane were then purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analysed by HPLC, as described previously (Hiraishi *et al.*, 1996).

The major cellular fatty acids of strains PCP11^T and PCP104 were iso-C_{15:0} (21.9 and 20.6%, respectively), summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 17.3 and 14.3%) and C_{15:0} (11.4 and 5.0%) (Table 2). The two isolates shared very similar fatty acid compositions but they differed from *R. agariperforans* KCTC 12369^T in the proportions of several fatty acids, especially C_{16:1}ω5c, C_{15:0}, iso-C_{15:1} F and iso-C_{16:0}. Menaquinone 7 (MK-7) was the major respiratory quinone of strains PCP11^T and PCP104, in line with all members of the family *Cytophagaceae*.

Chromosomal DNA extracted for 16S rRNA gene amplification was also used for determination of the G+C content. RNA in the DNA solution was removed by

incubation with a mixture of RNase A and T1 (each 20 U ml⁻¹) at 30 °C for 1 h. The G+C content was analysed as described by Mesbah *et al.* (1989) using reversed-phase HPLC. The DNA G+C contents of strains PCP11^T and PCP104 were 39.6 and 41.9 mol%, respectively. These values were significantly lower than that reported for *R. agariperforans* KMM 3525^T (44.5 mol%; Nedashkovskaya *et al.*, 2003).

Because of the rather low level of 16S RNA gene sequence similarity between strains PCP11^T and PCP104 and *R. agariperforans* KCTC 12369^T, we did not perform DNA–DNA hybridization experiments.

On the basis of phenotypic characteristics and phylogenetic inference, strains PCP11^T and PCP104 should be placed in the genus *Reichenbachiella* within a novel species, for which the name *Reichenbachiella faecimaris* sp. nov. is proposed. On the basis of the data obtained in this study, emended descriptions of the genus *Reichenbachiella* and *Reichenbachiella agariperforans* are also proposed.

Emended description of the genus *Reichenbachiella* Nedashkovskaya *et al.* 2005

The description of the genus *Reichenbachiella* is as given by Nedashkovskaya *et al.* (2005) with the following modifications and additions. Members of some species produce carotenoid and flexirubin-type pigments. The predominant fatty acids are iso-C_{15:0} and summed feature 4 (C_{16:1}ω7c

Table 1. Differential characteristics of strains PCP11^T and PCP104 and *R. agariperforans* KCTC 12369^T

Strains: 1, strain PCP11^T; 2, strain PCP104; 3, *R. agariperforans* KCTC 12369^T. All data are from this study unless indicated. All strains are strictly aerobic, Gram-staining-negative rods, motile by gliding and oxidase- and catalase-positive. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3
Colony pigmentation	Beige	Beige	Orange
Colonies sunken into the agar	–	–	+
Production of:			
Flexirubin pigments	–	–	+
Carotenoid pigments	–	–	+
Ranges for growth			
NaCl concentration (w/v, %)	1–5	1–5	1–6*
Temperature (°C)	5–35	5–35	4–35*
pH	5.5–8.5	5.5–8.5	5.5–10.0*
Hydrolysis of:			
Agar	–	–	+
Gelatin	–	–	+*
Casein	+	+	–
Alginate	–	–	+
Tween 20	–	–	+
DNA	–	–	+
Production of acid from:			
D-Xylose	–	–	+
D-Galactose	+	+	–
Maltose	+	+	w
Sucrose	+	–	w
Trehalose	+	+	–
Inulin	+	–	–
Gentiobiose	+	+	–
L-Fucose	–	–	+
Assimilation of:			
D-Mannose	–	–	+
D-Ribose	w	w	–
Glycogen	+	+	–
Phenylacetate	+	+	–
Lactate	w	w	–
L-Proline	+	+	–
Enzyme activity			
Cystine arylamidase	–	–	+
Naphthol-AS-BI-phosphohydrolase	+	+	w
α-Galactosidase	–	–	+
β-Galactosidase (2-naphthyl-β-D-galactopyranoside)	–	–	+
DNA G + C content (mol%)	39.6	41.9	44.5*

*Data from Nedashkovskaya *et al.* (2003).

and/or iso-C_{15:0} 2-OH). The DNA G + C content is approximately 39.6–44.5 mol%. As determined by 16S rRNA gene sequence analysis, the genus *Reichenbachiella* is a member of the family *Cytophagaceae*, phylum *Bacteroidetes*.

Emended description of *Reichenbachiella agariperforans* Nedashkovskaya *et al.* 2005

The description is as given by Nedashkovskaya *et al.* (2003, 2005) with the following modifications and additions.

Utilizes D-glucose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, sucrose, adipate, malate, acetate, L-serine, L-histidine and 2-ketogluconate; does not utilize L-arabinose, caprate, citrate, phenylacetate, L-rhamnose, D-ribose, inositol, itaconate, suberate, malonate, lactate, L-alanine, 5-ketogluconate, glycogen, salicin, melibiose, L-fucose, D-sorbitol, propionate, valerate, 3-hydroxybutyrate, 3-hydroxybenzoate, 4-hydroxybenzoate or L-proline (API 32GN and API 20NE galleries). Carotenoid and flexirubin-type pigments are produced.

Table 2. Cellular fatty acid compositions of strains PCP11^T and PCP104 and *R. agariperforans* KCTC 12369^T

Strains: 1, strain PCP11^T; 2, strain PCP104; 3, *R. agariperforans* KCTC 12369^T. All data are from this study. Values are percentages of total fatty acids; fatty acids that amounted to <1% of the total fatty acids in all strains are not shown. tr, Trace (<1% of the total); –, not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3
Saturated			
C _{12:0}	1.2	1.3	tr
C _{13:0}	1.1	tr	tr
C _{14:0}	2.3	3.2	1.8
C _{15:0}	11.4	5.0	–
C _{16:0}	2.6	5.8	4.0
C _{18:0}	1.6	4.9	2.0
Unsaturated			
C _{15:1ω6c}	2.1	1.9	tr
C _{16:1ω5c}	5.3	7.4	23.4
Branched chain			
iso-C _{14:0}	2.2	3.0	tr
iso-C _{15:0}	21.9	20.6	19.7
iso-C _{15:1} F	6.8	5.3	–
iso-C _{16:0}	2.9	4.6	tr
iso-C _{16:0} 3-OH	1.5	2.6	tr
iso-C _{17:0} 3-OH	1.2	1.4	1.9
Hydroxy			
C _{15:0} 2-OH	3.0	1.7	tr
C _{16:0} 2-OH	tr	1.5	1.6
Summed feature 4*	17.3	14.3	22.8
Unknown ECL 14.97	8.3	4.5	12.0

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 comprised iso-C_{15:0} 2-OH and/or C_{16:1ω7c}.

Description of *Reichenbachiella faecimaris* sp. nov.

Reichenbachiella faecimaris (fae'ci.ma'ris. L. n. *faex*, *faecis* sediment; L. n. *mare*, -is the sea; N.L. gen. n. *faecimaris* of the sediment of the sea).

Cells are Gram-staining-negative, strictly aerobic, oxidase- and catalase-positive, straight rods, approx. 0.3–0.5 µm in diameter and 3.0–5.0 µm long, motile by gliding. Endospores are not formed. Colonies are beige and circular with regular edges, approx. 1.0–2.0 mm in diameter after 3 days of incubation at 28 °C on MA. Colonies are not sunken into the agar. Growth occurs at 5–35 °C (optimum, 28–30 °C) and at pH 5.5–8.5 (optimum, pH 7.0–7.5). Requires sodium ions for growth; grows in the presence of 1–5% (w/v) NaCl (optimum, 3%). Carotenoid and flexirubin-type pigments are not produced. Casein, starch (soluble) and aesculin are hydrolysed, but gelatin, urea, alginate, DNA, Tweens 20, 40, 60 and 80, CM-cellulose, filter paper, starch (from rice), chitin and agar are not. Nitrate is not reduced, indole and H₂S are not produced

and D-glucose is not fermented. Utilizes D-glucose, N-acetylglucosamine, maltose, adipic acid, phenylacetic acid, sucrose, maltose, sodium acetate, L-alanine, glycogen, L-serine, L-histidine, potassium 2-ketogluconate and L-proline; does not utilize potassium gluconate, caprate, L-arabinose, D-mannose, citrate, L-rhamnose, inositol, itaconate, suberate, malonate, potassium 5-ketogluconate, salicin, melibiose, L-fucose, D-sorbitol, propionate, valerate, 3-hydroxybutyrate or 4-hydroxybenzoate (API 32GN and API 20NE galleries). Malate, D-ribose, lactate and 3-hydroxybenzoate are weakly utilized. Acid is produced from D-galactose, D-glucose, N-acetylglucosamine, amygdalin, aesculin ferric citrate, cellobiose, maltose, trehalose, glycogen, gentiobiose and potassium 2-ketogluconate. Production of acid from sucrose and inulin is strain-dependent. Acid is not produced from the other substrates in the API 50 CH gallery. Alkaline phosphatase, β-galactosidase (4-nitrophenyl β-D-galactopyranoside), esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase (weak) and N-acetyl-β-glucosaminidase activities are present; arginine dihydrolase, urease, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase (2-naphthyl β-D-galactopyranoside), β-glucuronidase, β-glucosidase (6-bromo-2-naphthyl β-D-glucopyranoside), α-mannosidase and α-fucosidase activities are absent (API 20NE and API ZYM galleries). Susceptible to (µg per disc) ampicillin (10), chloramphenicol (25), erythromycin (15), mitomycin C (50), streptomycin (10) and tetracycline (30), but resistant to gentamicin (10), kanamycin (30) and penicillin G (10). The major respiratory quinone is MK-7. The major cellular fatty acids are iso-C_{15:0}, summed feature 4 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) and C_{15:0}. The DNA G + C content is 39.6–41.9 mol% (HPLC).

The type strain is PCP11^T (=KACC 14523^T =JCM 16588^T), isolated from a tidal flat of the Yellow Sea, Korea.

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