Perlucidibaca piscinae gen. nov., sp. nov., a freshwater bacterium belonging to the family *Moraxellaceae*

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A freshwater bacterium, designated IMCC1704^T, was isolated from a eutrophic pond. The strain was Gram-negative, oxidase-positive, catalase-negative, chemoheterotrophic and facultatively aerobic with cells that were motile rods with a single polar flagellum. Based on 16S rRNA gene sequence similarity analyses, the novel strain was most closely related to the genera *Alkanindiges* (91.7 %), *Acinetobacter* (89.0–91.2 %), *Moraxella* (87.9–90.1 %), *Psychrobacter* (87.2–89.5 %) and *Enhydrobacter* (87.8 %). Phylogenetic trees generated using 16S rRNA gene sequences showed that the novel isolate belonged to the family *Moraxellaceae* of the class *Gammaproteobacteria* and formed a distinct phyletic lineage within the family. The DNA G+C content of the strain was 63.1 mol% and the predominant constituents of the cellular fatty acids were $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (21.2 %), $C_{18:1}\omega7c$ (12.8 %) and $C_{12:0}$ 3-OH (12.3 %). These chemotaxonomic properties, together with several phenotypic characteristics, differentiated the novel strain from other members of the family *Moraxellaceae.* From the taxonomic data, which revealed the distant relationship of the new strain to the related genera, the strain should be classified as a novel genus and species in the family *Moraxellaceae*, for which the name *Perlucidibaca piscinae* gen. nov., sp. nov. is proposed. The type strain of *Perlucidibaca piscinae* sp. nov. is IMCC1704^T (=KCCM 42363^T=NBRC 102354^T).

The family *Moraxellaceae* of the order *Pseudomonadales* in the class *Gammaproteobacteria* was proposed by Rossau *et al.* (1991) based on comprehensive DNA–rRNA hybridization results. The family currently comprises five recognized genera: *Moraxella* (Lwoff, 1939), *Acinetobacter* (Brisou & Prévot, 1954), *Psychrobacter* (Juni & Heym, 1986), *Enhydrobacter* (Staley *et al.*, 1987) and *Alkanindiges* (Bogan *et al.*, 2003). Because the members of the family *Moraxellaceae* are widely distributed in diverse environments and are of clinical importance, many novel species, especially within the genera *Acinetobacter* and *Psychrobacter*, have been isolated and classified recently (Bakermans *et al.*, 2006; Bozal *et al.*, 2003; Carr *et al.*, 2003; Jung *et al.*, 2005; Nemec *et al.*, 2001, 2003; Yoon *et al.*, 2005b). The present study focuses on the description of strain IMCC1704^T that was isolated from a freshwater pond (Song *et al.*, 2007). Based on the taxonomic data collected in this study, we propose the inclusion of the strain in a new genus and novel species within the family *Moraxellaceae*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC1704^T is DQ664237.

An electron micrograph of a cell of strain IMCC1704^T is available with the online version of this paper.

Strain IMCC1704^T was isolated from an artificial freshwater pond located inside Inha University, Korea, by a standard dilution plating method on R2A agar (Reasoner & Geldreich, 1985; Difco) plates. After incubating the agar plates aerobically at 20 °C for 5 days, strain IMCC1704^T was purified as single colonies and subsequently stored at −80 °C as 10 % (v/v) glycerol suspensions. After the optimum growth temperature of the strain was determined, cultures were routinely maintained on R2A agar at 30 °C.

DNA extraction, PCR and sequencing of the 16S rRNA gene were performed as described previously (Cho & Giovannoni, 2003) and almost complete 16S rRNA gene sequences (1467 bp) of strain IMCC1704^T were obtained. Preliminary sequence comparisons with 16S rRNA gene sequences held in the GenBank database showed that the novel strain belonged to the family *Moraxellaceae* in the class *Gammaproteobacteria*. The 16S rRNA gene sequences of the novel strain were aligned using the ARB software package (Ludwig *et al.*, 2004) and 1269 unambiguously aligned nucleotide positions were used for phylogenetic analyses in PAUP 4.0 beta 10 (Swofford, 2002). Phylogenetic trees were generated by the neighbour-joining (Saitou & Nei, 1987) method with Jukes–Cantor distance (Jukes & Cantor, 1969), maximum-parsimony (Fitch, 1971) and

maximum-likelihood (Felsenstein, 1981) algorithms. The resultant neighbour-joining and maximum-parsimony trees were evaluated by bootstrap analysis based on 1000 resamplings.

Sequence comparisons based on the multiple alignment in the ARB database, Ribosomal Database Project (RDP-II) and BLASTN search results showed that the most closely related cultured species with respect to strain IMCC1704^T was '*Alkanindiges hongkongensis*' HKU9 (92.5 % gene sequence similarity), which was described by Woo *et al.* (2005) but the name has not yet been validly published. Strain IMCC1704^T showed the highest 16S rRNA gene sequence similarity with the recognized species *Alkanindiges illinoisensis* DSM 15370^T (91.7 %), followed by *Acinetobacter calcoaceticus* ATCC 23055^T (91.2 %) and *Acinetobacter parvus* LMG 21765^T (91.0 %). Strain IMCC1704^T was moderately related to the genera of the family *Moraxellaceae* with relatively low 16S rRNA gene sequence similarities: *Alkanindiges*, 91.7 %; *Acinetobacter*, 89.0–91.2 %; *Moraxella*, 87.9–90.1 %; *Psychrobacter*, 87.2–89.5 % and *Enhydrobacter*, 87.8 %. No other recognized species exceeded 92.0 % 16S rRNA gene sequence similarity to strain IMCC1704^T. In all of the phylogenetic trees generated in this study, strain IMCC1704^T, the uncultured freshwater bacteria 156ds20 (GenBank accession no. AY212607; Simpson *et al.*, 2004) and 216ds20 (AY212663; Simpson *et al.*, 2004) formed an independent monophyletic lineage with 96–100 % bootstrap support for a position within the family *Moraxellaceae* (Fig. 1). This lineage formed a larger clade with the genera *Acinetobacter* and *Alkanindiges* in all of the phylogenetic trees; however, the phylogenetic relationship between strain IMCC1704^T and the genera *Acinetobacter* and *Alkanindiges* was distinct. The results of phylogenetic analyses revealed that strain IMCC1704^T could not be associated with any of the known genera in the family. Therefore, strain IMCC1704^T was considered to represent a new genus in the family *Moraxellaceae*.

For phenotypic characterizations, strain IMCC1704^T was routinely grown on R2A agar at 30 °C, unless otherwise specified. The type species of the most closely related genera *Acinetobacter* and *Alkanindiges* were also phenotypically characterized. *Alkanindiges illinoisensis* DSM 15370^T and *Acinetobacter calcoaceticus* ATCC 23055^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection and the Korean Culture and Type Collection (KCTC), respectively. Both type strains obtained from the culture collections were grown on R2A agar at 25 °C, unless otherwise indicated. Cell morphology and size were examined by transmission electron microscopy (CM200; Philips) and phase-contrast microscopy (80i; Nikon) using a 3 day culture. For electron microscopy, cells were washed twice with sodium cacodylate buffer and negatively stained with 2 % phosphotungstic acid (pH 7.0–7.2) on Formvar-coated copper grids. Colony morphology, size and colour were examined from cultures grown aerobically for 5 days. Motility based on flagella was tested from wet mounts using a 3 day culture. The growth temperature range and optimum were tested on R2A agar at 4, 10, 15, 20, 25, 30, 37 and 42 °C. The pH range and optimum were examined on R2A at pH values from pH 4.0 to 12.0 (at intervals of 1.0 pH units), adjusted with 0.1 M HCl and 0.1 M NaOH. The optimum NaCl concentration for growth was determined on R2A agar supplemented with 0–15 % NaCl (at intervals of 0.5 % from 0–5.0 %, 7.5 %, 10.0 %, 12.5 % and 15.0 %, w/v). The ranges and optima of temperature, pH and NaCl concentration for growth were monitored for 2 weeks. Anaerobic growth was tested using both the MGC anaerobic system (Mitsubishi Gas Chemical company, Inc.) and the Anaerocult C mini (EM Science). The catalase test was performed by the addition of 3.0 % hydrogen peroxide to fresh colonies and oxidase activity was determined using Kovacs' solution (Kovacs, 1956). Other biochemical tests were carried out with API 20NE

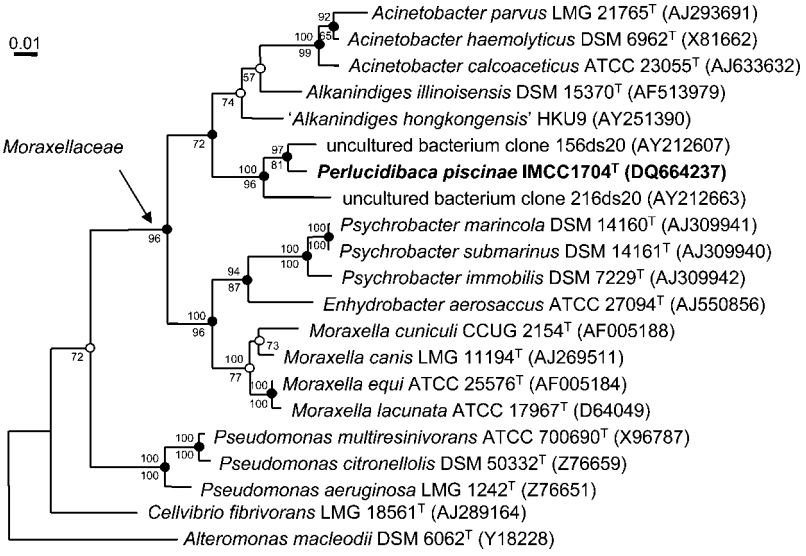

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing relationships between strain IMCC1704^T and representatives of the family *Moraxellaceae*. Bootstrap percentages (above 50 %) from both neighbour-joining (above nodes) and maximum-parsimony (below nodes) are shown. The closed and open circles at each node indicate nodes recovered reproducibly by all treeing methods or by two treeing methods, respectively. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics that differentiate strain IMC1704^T from the genera of the family *Moraxellaceae*

Taxa: 1, strain IMCC1704^T; 2, *Acinetobacter* (*n*=17, data taken collectively from this study and Bouvet & Grimont, 1986; Carr *et al.*, 2003; Juni & Bøvre, 2005; Nemec *et al.*, 2001, 2003; Nishmura *et al.*, 1988); 3, *Alkanindiges* (*n*=1, data from this study and Bogan *et al.*, 2003); 4, *Psychrobacter* (*n*=27, data taken collectively from Bakermans *et al.*, 2006; Heuchert *et al.*, 2004; Jung *et al.*, 2005; Juni & Bøvre, 2005; Romanenko *et al.*, 2004; Yoon *et al.*, 2005a, b); 5, *Enhydrobacter* (*n*=1, Staley *et al.*, 1987); 6, *Moraxella* (*n*=17, data taken collectively from Angelos *et al.*, 2007; Juni & Bøvre, 2005; Kodjo *et al.*, 1995; Vandamme *et al.*, 1993; Xie & Yokota, 2005). +, Present in 90 % or more of the species; −, absent in 90 % or more of the species; V, present in 11–89 % of the species, ND, no data available; W, weakly positive.

Characteristic	1	2	3	4	5	6
Flagellation	+	−	−	−	−	−
Growth at 37 °C	+	+	+	V	+	+
Anaerobic growth	+	−*	−	−	+	−†
Oxidase	+	−	−‡	+	+	+
Catalase	−	+†	+‡	+	+	+
Acid production from glucose	−	V	−‡	+	ND	−
Nitrate reduction	W	V	+‡	V	+	V
Indole production	+	−†	−‡	−	−	−
Major quinone	Q-8	Q-8‡, Q-9§	Q-8‡	Q-8	ND	Q-8‖
DNA G+C content (mol%)	63.1	40–46	46.2‡	42–50	66	40–49.6

*Data for *Acinetobacter calcoaceticus*, *Acinetobacter schindleri* and *Acinetobacter ursingii*.
†Data for *M. boevrei*, *M. lacunata* and *M. lincolnii*.
‡Data obtained from this study for *Acinetobacter calcoaceticus* ATCC 23055^T or *Alkanindiges illinoisensis* DSM 15370^T.
§Data for *Acinetobacter radioresistens*.
‖Data for *M. oblonga*.

Table 2. Biochemical characteristics and carbon source utilization patterns that differentiate strains IMCC1704^T, *Acinetobacter calcoaceticus* ATCC 23055^T and *Alkanindiges illinoisensis* DSM 15370^T

Strains: 1, IMCC1704^T; 2, *Acinetobacter calcoaceticus* ATCC 23055^T; 3, *Alkanindiges illinoisensis* DSM 15370^T. +, Positive; −, negative; W, weakly positive. All data were obtained in the present study. All the strains were negative for acid production from glucose, for activities of arginine dihydrolase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase and for utilization of methanol, ethanol, DL-glyceraldehyde, D-cellobiose, D-lactose, melibiose, D-melezitose, adonitol, succinic acid, L-glutamic acid and L-leucine. All the strains were positive for esterase (C4) and esterase lipase (C8) activities and for utilization of L-proline and were weakly positive for aesculin hydrolysis.

Characteristic	1	2	3
API 20NE			
Nitrate reduction	W	+	+
Indole production	+	−	−
Gelatin liquefaction	−	−	W
Urease activity	−	+	+
API ZYM			
Alkaline phosphatase	+	+	−
Lipase (C14)	−	+	+
Leucine arylamidase	−	+	−
Valine arylamidase	−	+	−
Cystine arylamidase	−	+	−
Naphthol-AS-BI-phosphohydrolase	−	+	+
Carbon source utilization			
Methylamine, D-ribose, D-arabinose, *N*-acetyl-D-glucosamine, D-glucose, rhamnose, maltose, sucrose, trehalose, D-raffinose, arabitol, D-mannitol, *myo*-inositol, D-sorbitol, D-xylitol, citric acid, malonic acid, L-arginine, L-glycine, L-serine, L-lysine	+	−	−
D-Xylose, D-galactose, D-mannose, pyruvic acid, L-histidine, L-ornithine, D-glucosamine	+	+	−
Glycerol, gluconic acid, itaconic acid,	+	−	+
D-Fructose, glucuronic acid, L-alanine	−	+	−
Propionic acid	−	+	+

and API ZYM (bioMérieux) kits following the manufacturer's instructions. Sole carbon source utilization tests were performed using custom-made 48-well microplates containing 47 different carbon compounds at a final concentration of 0.2 % (w/v or v/v), according to Choo *et al.* (2007) except that microplates were inoculated with bacterial suspensions in phosphate-buffered saline (pH 7.4). Cells at the exponential growth phase were harvested and cell densities were adjusted to approximately 2×10^3 cells ml^{-1} in phosphate-buffered saline (pH 7.4). A 1 ml sample of cell suspension was inoculated per well and the microplates were incubated at 30 °C for strain IMCC1704^T and 25 °C for strains DSM 15370^T and ATCC 23055^T. After incubation of the microplates for 1 week, cellular growth and purity were checked by DAPI-stained epifluorescence microscopy. The DNA G+C content (Mesbah *et al.*, 1989) was analysed by using HPLC with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco). Cellular fatty acid methyl esters of strains IMCC1704^T, DSM 15370^T and ATCC 23055^T were prepared from the cultures grown on R2A agar at 30 °C for 3 days and were analysed according to the instructions of the Microbial Identification System (MIDI) by the Korean Culture Center of Microorganisms (KCCM). Respiratory quinones were analysed using a reverse-phase HPLC (Komagata & Suzuki, 1987).

Strain IMCC1704^T was Gram-negative, chemoheterotrophic, facultatively aerobic, catalase-negative and oxidase-positive. Cells were motile short-rods that had a single polar flagellum (see Supplementary Figure S1 in IJSEM online). Detailed results of the phenotypic and biochemical tests are given in the species description and in Tables 1, 2 and 3. Strain IMCC1704^T could be differentiated from the other genera of the family *Moraxellaceae* by several phenotypic characteristics and the DNA G+C content (Table 1). The DNA G+C content of strain IMCC1704^T was 63.1 mol%, which was 13–23 mol% higher than that of other members of the family *Moraxellaceae*, except for *Enhydrobacter aerosaccus*. The biochemical characteristics and carbon source utilization patterns also clearly differentiated strain IMCC1704^T from the type strains of the type species of the genera *Alkanindiges and Acinetobacter* (Table 2). The major fatty acid constituents of strain IMCC1704^T were $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (21.2 %), $C_{18:1}\omega7c$ (12.8 %), $C_{12:0}$ 3-OH (12.3 %), $C_{12:0}$ (10.1 %) and $C_{18:1}\omega9c$ (9.2%), and they were different from those of *Alkanindiges illinoisensis* DSM 15370^T and *Acinetobacter calcoaceticus* ATCC 23055^T.

It is evident from the low 16S rRNA gene sequence similarity (<92 %), the formation of an independent phyletic line in the phylogenetic analyses (Fig. 1) and the differential phenotypic characteristics (Tables 1, 2 and 3) that strain IMCC1704^T cannot be assigned to any of the known genera in the family *Moraxellaceae*. Conclusively, based on the taxonomic results in this study, strain IMCC1704^T should be classified as a novel species within a new genus, for which the name *Perlucidibaca piscinae* gen. nov., sp. nov. is proposed.

Description of *Perlucidibaca* gen. nov.

Perlucidibaca (Per.lu.ci.di.ba′ca. L. adj. *perlucidus* transparent, pellucid; L. fem. n. *baca* a small round fruit, a berry; N.L. fem. n. *Perlucidibaca* a transparent berry).

Gram-negative. Oxidase-positive and catalase-negative. Chemoheterotrophic and facultatively aerobic. Anaerobic growth is similar to aerobic growth. Cells are short rods that are motile by a polar flagellum. Indole is produced. Nitrate reduction is weakly positive. Acid is not produced from glucose fermentation. Predominant cellular fatty acids are $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{18:1}\omega7c$, $C_{12:0}$ 3-OH and $C_{12:0}$. The DNA G+C content is 63.1 mol%. The major respiratory quinone is Q-8. Phylogenetically, the genus belongs to the family *Moraxellaceae*. The type species is *Perlucidibaca piscinae*.

Description of *Perlucidibaca piscinae* sp. nov.

Perlucidibaca piscinae (pis.ci′nae. L. gen. n. *piscinae* of a fish-pond).

The description is the same as that for the genus, with the following additional properties. Cells in the exponential phase are short rods, 0.7–1.2 μm long and 0.5–0.7 μm wide. Colonies on R2A agar are circular, convex, smooth, butyrous and transparent with an entire margin. Colony

Table 3. Cellular fatty acid content (%) of strain IMCC1704^T, *Acinetobacter calcoaceticus* ATCC 23055^T and *Alkanindiges illinoisensis* DSM 15370^T

Strains: 1, IMCC1704^T; 2, *Acinetobacter calcoaceticus* ATCC 23055^T; 3, *Alkanindiges illinoisensis* DSM 15370^T. −, Not detected. All the strains were grown on R2A agar at 30 °C for 3 days. Only fatty acids representing at least 1 % of the total cellular fatty acids of at least one of the strains are shown.

Fatty acid	1	2	3
$C_{10:0}$	3.4	0.2	4.9
$C_{12:0}$	10.1	5.5	3.6
$C_{14:0}$	3.1	0.9	1.9
$C_{15:0}$	0.5	1.3	0.6
$C_{16:0}$	5.8	14.7	16.9
$C_{17:0}$	0.7	3.4	−
$C_{18:0}$	2.7	1.7	0.9
$C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH	21.2	20.4	42.7
$C_{17:1}\omega8c$	5.5	5.6	1.1
$C_{18:1}\omega7c$	12.8	2.0	0.6
$C_{18:1}\omega9c$	9.2	28.9	10.3
$C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$	−	−	1.3
$C_{10:0}$ 2-OH	1.5	−	1.7
$C_{10:0}$ 3-OH	0.6	−	1.3
$C_{12:0}$ 2-OH	0.4	2.7	1.0
$C_{12:0}$ 3-OH	12.3	5.3	9.6
iso-$C_{17:0}$	0.9	1.1	−
Unknown peak 12.488	1.4	0.6	0.8
Unknown peak 10.928	−	3.0	−

size is 0.5–1.0 mm after incubation on R2A at 30 °C for 5 days. Grows at 8–37 °C (optimally at 30 °C), but not at 4 and 42 °C. Growth occurs at pH 6–10 and with 0–1 % NaCl; optimum at pH 7.0 and without NaCl. Biochemical characteristics and carbon source utilization patterns are shown in Table 2. The cellular fatty acid content is listed in Table 3.

The type strain, IMCC1704^T (=KCCM 42363^T=NBRC 102354^T), was isolated from an artificial freshwater pond located inside Inha University, Korea.

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