Litoricola marina sp. nov.

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A Gram-negative, non-pigmented, non-motile, chemoheterotrophic marine bacterium, designated strain IMCC2782^T, was isolated from a surface seawater sample of the Yellow Sea, Korea. On the basis of 16S rRNA gene sequence analysis and phenotypic and genotypic characterization, strain IMCC2782^T is shown to represent a novel species of the genus *Litoricola*. Strain IMCC2782^T grew at 15–37 °C and tolerated 7.5 % NaCl. Based on 16S rRNA gene sequences, strain IMCC2782^T was related most closely to the type strain of *Litoricola lipolytica* (97.1 % similarity) but showed less than 90 % similarity to the type strains of other bacterial species. The predominant fatty acids were mono-unsaturated $C_{18:1}$ and $C_{16:1}$. The G+C content of the DNA of strain IMCC2782^T was 59.6 mol%. A low level of DNA–DNA relatedness (28.1 %) together with several phenotypic characteristics, including enzyme activities, served to differentiate strain IMCC2782^T from the type strain of *L. lipolytica*. Therefore, strain IMCC2782^T is considered to represent a novel species of the genus *Litoricola*, for which the name *Litoricola marina* sp. nov. is proposed. The type strain is IMCC2782^T (=KCTC 22683^T =NBRC 105824^T).

The genus *Litoricola* (Kim *et al.*, 2007) in the family *Litoricolaceae* (Kim *et al.*, 2007), order *Oceanospirillales*, comprises (at the time of writing) just one recognized species, *Litoricola lipolytica*. The type strain of *L. lipolytica* was isolated from coastal seawater of the East Sea of Korea and was characterized as Gram-negative, non-motile, chemoheterotrophic and facultatively aerobic, forming very small colonies and short rod-shaped cells. In the present study, we describe another coastal seawater strain, designated IMCC2782^T, which was isolated from the Yellow Sea. Phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequences indicated that strain IMCC2782^T represents a novel species of the genus *Litoricola*.

Strain IMCC2782^T was isolated from a seawater sample collected from the surface (1 m depth) of the Yellow Sea (near Yeongjong Do, Incheon, Korea; 37° 27′ 44″ N 126° 29′ 59″ E). A serially diluted seawater sample was spread on to oligotrophic medium, R2A agar (BD Difco), diluted 1 : 10 (v/v) with aged seawater (1/10R2A), and the plates were incubated aerobically at 20 °C for 1 month. After the optimum growth temperature of the strain was determined, bacterial cultures were routinely maintained on marine agar 2216 (MA; BD Difco) or in marine broth 2216 (MB; BD Difco) at 25 °C and stored as glycerol suspensions (20 %, v/v) at −80 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC2782^T is FJ943234.

Bacterial DNA extraction, PCR amplification and 16S rRNA gene sequencing were performed as described by Cho & Giovannoni (2003). The almost-complete 16S rRNA gene sequence (1475 bp) of strain IMCC2782^T was aligned with its nearest relatives by using the ARB software package (Ludwig *et al.*, 2004) and PAUP* 4.0 beta (Swofford, 2002) as described by Cho & Giovannoni (2003). Comparative sequence analyses in the ARB database were also confirmed via the EzTaxon server (Chun *et al.*, 2007). Based on 16S rRNA gene sequence comparisons, strain IMCC2782^T was shown to be related most closely to *L. lipolytica* KCCM 42360^T (97.1 % similarity); the novel strain did not exhibit more than 90 % 16S rRNA gene sequence similarity to the type strain of any other recognized species. To investigate the phylogeny of strain IMCC2782^T, 1233 nucleotide positions that could be unambiguously aligned were used to create phylogenetic trees with the neighbour-joining (Saitou & Nei, 1987) (with the Jukes–Cantor distance formula; Jukes & Cantor, 1969), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. The robustness of the neighbour-joining and maximum-parsimony trees was checked by bootstrap analyses based on 1000 resamplings. In all the phylogenetic trees generated in this study (Fig. 1), strain IMCC2782^T and *L. lipolytica* KCCM 42360^T formed a robust monophyletic clade with high bootstrap support (100 %), indicating that the novel strain is a member of the genus *Litoricola*.

DNA–DNA relatedness, a basis for bacterial species demarcation (Wayne *et al.*, 1987), was determined

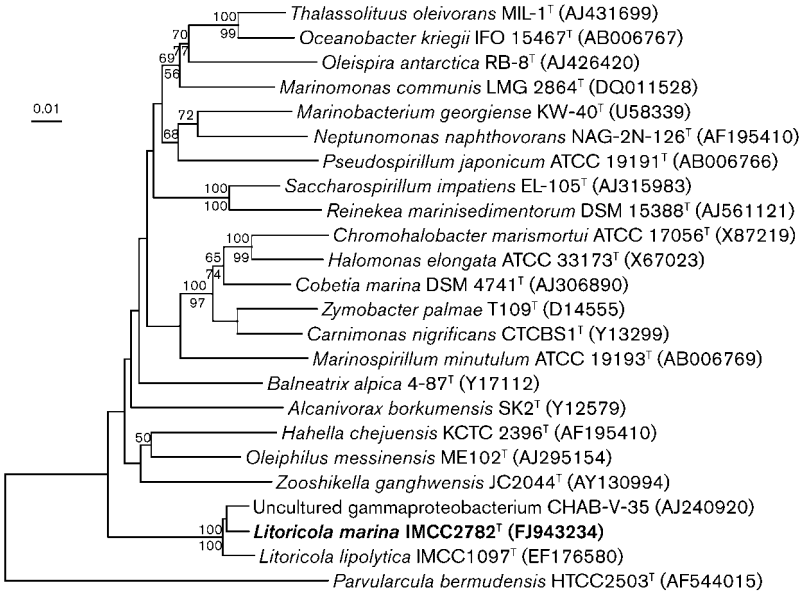

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain IMCC2782^T and its relatives in the order *Oceanospirillales*. Bootstrap values (above 50 %) from both neighbour-joining (above nodes) and maximum-parsimony (below nodes) methods are presented. Nodes recovered reproducibly by all treeing methods (filled circles) or by two treeing methods (open circles) are indicated. Bar, 0.01 substitutions per nucleotide position.

experimentally because the level of 16S rRNA gene sequence similarity between *L. lipolytica* KCCM 42360^T and strain IMCC2782^T was greater than 97.0 %. DNA–DNA hybridization was performed fluorometrically (Ezaki *et al.*, 1989) by using photobiotin-labelled DNA probes and microdilution wells. The level of DNA–DNA relatedness between strain IMCC2782^T and *L. lipolytica* KCCM 42360^T was 28.1 % (mean from five microdilution wells), suggesting that strain IMCC2782^T should be assigned to a genomic species separate from *L. lipolytica*.

For phenotypic characterization, strain IMCC2782^T was routinely grown on MA or in MB at 25 °C. The temperature range for growth was determined on the basis of colony formation on MA plates incubated at 4, 10, 15, 20, 25, 30, 37 and 42 °C. The pH range and optimum pH for growth were examined from pH 4.0 to 12.0 (at 0.5 pH unit intervals) in MB adjusted to the appropriate pH with 0.1 M HCl or 0.1 M Na_2CO_3. The range of and optimum NaCl concentrations for growth were determined in NaCl-free artificial seawater medium (Choo *et al.*, 2007) amended with 5.0 g peptone, 1.0 g yeast extract and various concentrations of NaCl (0–15 %, w/v). Cell size and morphology were determined by using phase-contrast microscopy and epifluorescence microscopy (Nikon 80i) of cells stained with 4′,6-diamidino-2-phenylindole (DAPI). Colony characteristics were examined after growing aerobic cultures on MA for 1 week. Anaerobic growth was tested on MA by using the MGC anaerobic system (Mitsubishi Gas Chemical Company) for 3 weeks. Tests for catalase and oxidase were performed by assessing bubble production in 3.0 % hydrogen peroxide solution and by using Kovacs' solution (Kovacs, 1956), respectively. Other biochemical tests and substrate oxidation tests were carried out by using API 20NE, API ZYM and API 50CH test strips (bioMérieux) by inoculating cells into ASW medium. For API 20NE, API ZYM and API 50CH analyses, *L. lipolytica* KCCM 42360^T was used as reference strain under the same culture conditions. The following antibiotics were tested by using the diffusion plate method: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg).

The phenotypic characteristics of strain IMCC2782^T are given in the species description below and in Table 1. Cells of strain IMCC2782^T were Gram-negative, chemoheterotrophic, facultatively anaerobic, oxidase- and catalase-positive, non-motile, non-pigmented short rods. A number of phenotypic characteristics, including enzyme activities via the API ZYM system, could be used to differentiate strain IMCC2782^T from *L. lipolytica* (Table 1).

The DNA G+C content was determined by using the HPLC method (Mesbah *et al.*, 1989). The G+C content of the genomic DNA of strain IMCC2782^T was 59.6 mol%, only 1.7 mol% different from that of *L. lipolytica* KCCM 42360^T, again suggesting that the two strains belong to the same genus. Cellular fatty acid methyl esters of strain IMCC2782^T were extracted from cultures grown on MA at 25 °C for 1 week, the same culture conditions as used for fatty acid analysis of *L. lipolytica* KCCM 42360^T. Fatty acids were analysed according to the Sherlock Microbial Identification System (MIDI).

The major cellular fatty acid constituents of strain IMCC2782^T were $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (41.1 %), $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (29.5 %), $C_{10:0}$ 3-OH (13.8 %), $C_{16:0}$ (6.1 %) and $C_{12:1}$ 3-OH (5.9 %), largely similar to the major fatty acid composition of *L. lipolytica* KCCM 42360^T except for the proportions of $C_{18:1}\omega7c$ and $C_{16:1}\omega7c$ (Table 2).

Table 1. Differential characteristics between strain IMCC2782^T and *Litoricola lipolytica* KCCM 42360^T

Unless indicated, data for *L. lipolytica* KCCM 42360^T were taken from Kim *et al.* (2007). +, Positive; −, negative; w, weakly positive. Cells of both strains were Gram-negative, facultatively aerobic, non-motile and oxidase-positive. In the API 20NE system, both strains were positive for β-galactosidase activity and negative for nitrate reduction, indole production, glucose fermentation, gelatin liquefaction, arginine dihydrolase and urease and gelatinase activities. In the API ZYM system, the two strains were positive for esterase lipase (C8) activity and negative for β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. In the API 50CH system, both strains were positive for acid production from glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-glucose, D-fructose, D-mannose, aesculin, ferric citrate and potassium 2-ketogluconate, weakly positive for acid production from D-galactose and D-arabitol and negative for acid production from inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, melezitose, raffinose, xylitol, gentiobiose, turanose and potassium 5-ketogluconate. Both strains were susceptible to chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin, tetracycline and vancomycin but resistant to ampicillin and penicillin G.

Characteristic	Strain IMCC2782^T	*L. lipolytica* KCCM 42360^T
Temperature range for growth (°C)	15–37	15–30
Growth with 10 % NaCl	−	+
Growth at pH 12.0	+	−
Catalase	+	−
Aesculin hydrolysis (API 20NE)	−	+*
Enzyme activities (API ZYM)		
Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase	+	−*
Lipase (C14), α-galactosidase	w	−*
Acid production from (API 50CH):		
L-Sorbose, sucrose, inulin, D-lyxose	+	−*
L-Rhamnose, dulcitol	+	w*
D-Mannitol, D-sorbitol, starch	w	−*
Glycogen	−	w*
DNA G+C content (mol%)	59.6	57.9

*Data from the present study.

Table 2. Cellular fatty acid compositions of strain IMCC2782^T and *L. lipolytica* KCCM 42360^T

Data for *L. lipolytica* KCCM 42360^T were taken from Kim *et al.* (2007). The two strains were grown under the same culture conditions (on MA at 25 °C for 1 week). Values are percentages of the total fatty acids; only those components accounting for at least 1 % of the total are shown. −, Not detected.

Fatty acid	Strain IMCC2782^T	*L. lipolytica* KCCM 42360^T
$C_{10:0}$	1.2	−
$C_{16:0}$	6.1	6.5
$C_{16:1}\omega7c$	29.5*	42.8†
$C_{18:1}\omega7c$	41.1‡	20.6
$C_{10:0}$ 3-OH	13.8	14.1
$C_{12:1}$ 3-OH	5.8	5.6

*Detected as $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$.
†Detected as $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.
‡Detected as $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

Based upon the formation of a robust monophyletic clade between strain IMCC2782^T and the type strain of *L. lipolytica* and similar chemotaxonomic characteristics (including DNA G+C content and major cellular fatty acids), strain IMCC2782^T should be assigned to the genus *Litoricola*. However, the low level of DNA–DNA relatedness (28.1 %), differences in several phenotypic properties (Table 1), the low level of 16S rRNA gene sequence similarity (97.1 %) (Stackebrandt & Ebers, 2006) and the phylogenetically distinct relationship (Fig. 1) between the two strains indicate that strain IMCC2782^T represents a novel species of the genus *Litoricola*, for which the name *Litoricola marina* sp. nov. is proposed.

Description of *Litoricola marina* sp. nov.

Litoricola marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine).

Cells are Gram-negative, oxidase- and catalase-positive, non-motile, non-pigmented, chemoheterotrophic and facultatively anaerobic. Grows better under aerobic than under anaerobic conditions. Cells are short rods (0.6–1.8 × 0.3–0.7 μm). After 1 week of incubation on MA at

25 °C, colonies are circular, convex, opaque, smooth, cream-coloured and 0.4 mm in diameter. Growth occurs at 15–37 °C (optimum, 25 °C), at pH 5.0–12.0 (optimum, pH 8.0) and in the presence of 1.0–7.5 % NaCl (optimum, 2.5–3.0 % NaCl). Positive for β-galactosidase (API 20NE), but negative for nitrate reduction, indole production, glucose fermentation, gelatin liquefaction, arginine dihydrolase and urease and gelatinase activities. Positive for the following enzyme activities (API ZYM): esterase lipase (C8), alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Oxidizes various carbon compounds under aerobic conditions. Detailed phenotypic characteristics are given in Table 1. Major cellular fatty acids are $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, $C_{10:0}$ 3-OH, $C_{16:0}$ and $C_{12:1}$ 3-OH. The DNA G+C content of the type strain is 59.6 mol%.

The type strain, IMCC2782^T (=KCTC 22683^T =NBRC 105824^T), was isolated from coastal seawater of the Yellow Sea, Korea.

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