Bacillus luteolus sp. nov., a halotolerant bacterium isolated from a salt field

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A novel Gram-stain-positive, motile, strictly aerobic bacterium, designated YIM 93174^T, was isolated from a salt field in Korea. Cells of this strain were rod-shaped and formed pale tangerine colonies and grew at pH 6.0–8.0 (optimal growth at pH 7.0), at 15–45 °C (optimum 28–37 °C) and at salinities of 0–10 % (w/v) NaCl (optimum 0–2 % NaCl). Some phenotypic characters allowed differentiation of strain YIM 93174^T from its nearest phylogenetic relatives. Comparative 16S rRNA gene sequence analysis showed that strain YIM 93174^T belongs to the genus *Bacillus*, exhibiting the highest level of sequence similarity with the type strain of *Bacillus humi* (95.7 %), followed by those of *Bacillus alkalitelluri*s (94.9 %) and *Bacillus litoralis* (94.5 %). The major fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The cell-wall peptidoglycan was of the A1γ type, containing *meso*-diaminopimelic acid as the diagnostic diamino acid. The genomic DNA G+C content was 36.9 mol% and the predominant respiratory quinone was MK-7. The major polar lipids of strain YIM 93174^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and two unknown phospholipids. On the basis of the evidence from this polyphasic study, strain YIM 93174^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus luteolus* sp. nov. is proposed, with YIM 93174^T (=DSM 22388^T =KCTC 13210^T =CCTCC AA 208068^T) as the type strain.

Since the genus *Bacillus* was first described by Cohn in 1872, the number of *Bacillus* species has increased considerably. It is one of the largest bacterial genera, including more than 200 species (Euzéby, 2010). Many *Bacillus* species are aerobic, spore-forming, halophilic or halotolerant, Gram-positive rods. The genus is considered to be ubiquitous, because *Bacillus* species have been isolated from a wide variety of aquatic and terrestrial environments, such as dust (Venkateswaran *et al.*, 2003), river-mouth sediments (Ruiz-García *et al.*, 2005), sandy soil (Lee *et al.*, 2008) and hypersaline lakes (Amoozegar *et al.*, 2009). During an investigation of the culturable bacterial community in sediments from a salt field in South Korea, a large number of bacterial strains were isolated. In this study, we characterized a new isolate, designated YIM 93174^T. Based on the results of a polyphasic study conducted according to the minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009), strain YIM 93174^T is proposed to represent a novel species of the genus *Bacillus*.

Strain YIM 93174^T was isolated from a salt field in Mokpo city, South Korea. For isolation, serial dilutions of the sample were spread on glucose-tryptone-yeast extract (GTY) medium (containing 5 % NaCl), described by Tang *et al.* (2010), and incubated at 37 °C for 3 weeks. Colonies were picked and restreaked repeatedly onto GTY medium until purity was confirmed. Strain YIM 93174^T was maintained on GTY agar slants at 4 °C and in 25 % (v/v) glycerol suspensions at –80 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (about 150 r.p.m.) using GTY broth (2 % NaCl, pH 7.0) at 37 °C for about a week.

Genomic DNA was extracted and purified according to the method described by Li *et al.* (2007). The 16S rRNA gene

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Abbreviation: *m*-Dpm, *meso*-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 93174^T is GQ925365.

Four supplementary figures and a supplementary table are available with the online version of this paper.

was amplified by PCR using primers 8-27f and 1523-1504r (Cui *et al.*, 2001) and PCR products were purified using a PCR purification kit (Sangon). The purified PCR product was sequenced directly on an ABI model 3730 automatic DNA sequencer by using a PRISM ready reaction dye primer cycle sequencing kit. The resulting sequence data were analysed with the BLASTN program of GenBank. Sequences were aligned using the CLUSTAL_X software (Thompson *et al.*, 1997) and the alignment was corrected manually. Phylogenetic analyses were performed using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) using MEGA version 4.0 (Tamura *et al.*, 2007). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method with 1000 replicates (Felsenstein, 1985). The genomic DNA G+C content was determined by the method of Mesbah *et al.* (1989) using DNA prepared according to Marmur (1961); DNA from *Escherichia coli* JM 109 was used as a control.

An almost-complete 16S rRNA gene sequence (1486 bp) of strain YIM 93174^T was obtained. Fig. 1 shows the relationship of the novel strain and its nearest phylogenetic relatives, based on a neighbour-joining analysis of 16S rRNA gene sequences. The results of 16S rRNA gene sequence comparison clearly demonstrated that strain YIM 93174^T was a member of the genus *Bacillus* and formed a distinct subclade with *Bacillus humi* LMG 22167^T, *Bacillus litoralis* SW-211^T and *Bacillus alkalitelluris* BA288^T. The 16S rRNA gene sequence similarity between strain YIM 93174^T and its closest neighbours, *B. humi* LMG 22167^T, *B. litoralis* SW-211^T and *B. alkalitelluris* BA288^T, was 95.7, 94.9 and 94.5 %, respectively. Topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed by neighbour-joining analysis (Fig. 1 and Supplementary Figs S1 and S2, available in IJSEM Online). The genomic DNA G+C content of strain YIM 93174^T was 36.9 mol%.

Cell morphology was determined using cultures grown for 6, 12, 24, 48 and 72 h on GTY agar medium supplemented with 2 % (w/v) NaCl at 37 °C and examined by transmission electron microscopy (H-800; Hitachi). Gram staining was carried out by the standard Gram reaction combined with the KOH lysis test (Cerny, 1978). MSM medium (Vaishampayan *et al.*, 2009) supplemented with 2 % NaCl was used to induce sporulation at 37 °C for 24 h and purified spores were detected by using malachite green staining (Smibert & Krieg, 1994). Cell motility was detected by the presence of turbidity throughout tubes containing semisolid medium (Leifson, 1960). Methyl red and Voges–Proskauer tests were performed as described previously (Smibert & Krieg, 1981). The temperature range for growth was determined by incubating cells for a week on GTY medium at 5–55 °C (at intervals of 5 °C). Growth was investigated at pH 4.0–10.0, at intervals of 1 pH unit, using the buffer system described by Xu *et al.* (2005). Growth in 0–20 % (w/v) NaCl, at intervals of 1 %, was tested by using ISP 4 medium as the basal medium. *B. humi* DSM 16318^T, *B. litoralis* DSM 16303^T and *B. alkalitelluris* DSM 16976^T

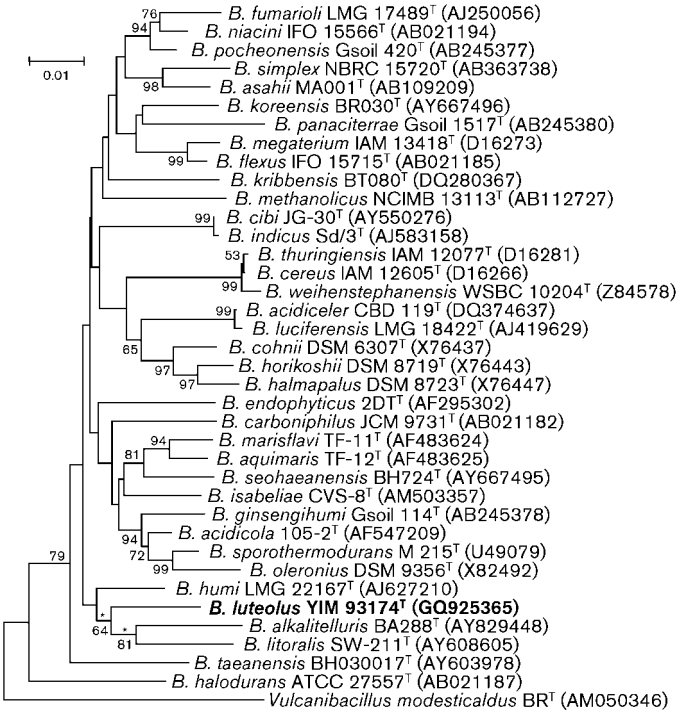

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis, reconstructed using the neighbour-joining method (Saitou & Nei, 1987), showing the interrelationship of strain YIM 93174^T and members of closely related species. Asterisks indicate branches that were also found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms (Supplementary Figs S1 and S2). Numbers at branching points refer to bootstrap values (percentages of 1000 resamplings; only values above 50 % are shown). The sequence of *Vulcanibacillus modesticaldus* BR^T was used as an outgroup. Bar, 1 % sequence divergence.

were used as reference strains for physiological and biochemical characteristics tests and they were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Catalase activity was determined by production of bubbles after addition of a drop of 3 % H_2O_2. Oxidase activity was observed by oxidation of tetramethyl-*p*-phenylenediamine. Hydrolysis of aesculin, dextrin, casein, gelatin, starch, urea and Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965). Enzyme activity and acid production tests were carried out using the API 50CHB, API 20E and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Utilization of different compounds as sole carbon or nitrogen and energy sources was tested as described by Carrasco *et al.* (2006). Antibiotic susceptibility was determined by the method of Williams (1967). Anaerobic growth was determined using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions.

The morphological characteristics of strain YIM 93174^T were distinctly different from those of *B. humi* DSM 16318^T, *B. litoralis* DSM 16303^T and *B. alkalitelluris* DSM 16976^T (Table 1). The detailed physiological and biochemical characteristics of the strain are given in the species description.

Peptidoglycan was purified and cell-wall amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates using the solvent systems of Schleifer & Kandler (1972). For fatty acid analysis, cells of strain YIM 93174^T was cultured on tryptic soy agar (TSA; BD) containing 2 % NaCl at 37 °C with shaking for 48 h. Analysis of cellular fatty acids was performed as described by Sasser (1990) using the Microbial Identification System (MIDI, Inc.). Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). Polar lipids were extracted, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1984). The peptidoglycan contained *meso*-diaminopimelic acid (*m*-Dpm), which is characteristic of the genus *Bacillus* [peptidoglycan type A1γ (*m*-Dpm direct, type A31); DSMZ, 2004]. The predominant menaquinone of the isolate was MK-7. Strain YIM 93174^T had a cellular fatty acid profile that contained major amounts of branched fatty acids and minor amounts of straight-chain and unsaturated fatty acids; the major cellular fatty acids were iso-$C_{15:0}$ (46.2 %), anteiso-$C_{15:0}$ (17.1 %) and iso-$C_{16:0}$ (13.7 %). The polar lipids of strain YIM 93174^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and two unknown phospholipids (Supplementary Fig. S3). The chemotaxonomic properties of strain YIM 93174^T [A1γ (*m*-Dpm direct, type A31) as peptidoglycan type; iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$ as major fatty acids; MK-7 as predominant menaquinone] are similar to those of *B. humi* DSM 16318^T, *B. litoralis* DSM 16303^T and *B. alkalitelluris* DSM 16976^T (Heyrman *et al.*, 2005; Yoon & Oh, 2005; Lee *et al.*, 2008). However, strain YIM 93174^T exhibited some differences from *B. humi* DSM 16318^T, *B. litoralis* DSM 16303^T and *B. alkalitelluris* DSM 16976^T in their fatty acid compositions (Supplementary Table S1), which were determined under the same laboratory conditions.

Thus, based on phenotypic (Table 1 and Supplementary Table S1) and phylogenetic (Fig. 1) differences between the novel strain and previously described species within the genus *Bacillus*, we consider that strain YIM 93174^T represents a novel species, for which we propose the name *Bacillus luteolus* sp. nov.

Description of *Bacillus luteolus* sp. nov.

Bacillus luteolus (lu.te.o'lus. L. masc. adj. *luteolus* yellowish, pale tangerine-coloured).

Cells are Gram-stain-positive, strictly aerobic, thin rods, 0.7–0.9 × 2.0–5.0 μm, motile by means of peritrichous flagella (Supplementary Fig. S4). Spherical endospores are produced in a terminal position. Colonies are 0.5–2 mm in diameter, circular, pale tangerine in colour, convex and opaque on GTY medium. Grows at 0–10 % (w/v) NaCl, with optimal growth at 0–2 % (w/v) NaCl. Growth occurs at 15–45 °C and at pH 6.0–8.0, with optimal growth at 28–37 °C and pH 7.0. Nitrate is not reduced to nitrite under anaerobic or aerobic conditions. Catalase is detected. Oxidase, methyl red and Voges–Proskauer tests are negative. Indole and H_2S are not produced. Dextrin and Tweens 20, 40 and 60 are hydrolysed, but casein, gelatin, starch, Tween 80 and aesculin are not hydrolysed. Acids are produced from potassium 5-ketogluconate (weakly), D-fructose, sucrose, turanose, D-xylose, glycerol (weakly) and glycogen (weakly), but not from L-arabinose, D-ribose, L-xylose, D-galactose, D-glucose, D-mannose, L-rhamnose, *myo*-inositol, D-mannitol, D-sorbitol, cellobiose, maltose, melibiose, trehalose, melezitose or raffinose (API 50CHB). In the API 20E system, positive for arginine dihydrolase and urease, but negative for *o*-nitrophenyl β-D-galactoside hydrolysis, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. In the API ZYM system, tests for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase are positive, but valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lysine decarboxylase and ornithine decarboxylase tests are negative. The following compounds are utilized as sole carbon or nitrogen sources: L-arginine, hypoxanthine, sodium citrate, lactose, L-rhamnose, D-xylose, D-ribose and glycerol. Susceptible to (μg per disc unless indicated) amikacin (30), ampicillin (10), ciprofloxacin (5), clindamycin (2), erythromycin (15), gentamicin (10), novobiocin (30), streptomycin (10), tobramycin (10), vancomycin (30), amoxicillin (10), chloramphenicol (30), netilmicin sulfate (30), norfloxacin (10), penicillin (10 U), rifampicin (5), trimethoprim

Table 1. Characteristics that differentiate strain YIM 93174^T from the type strains of closely related species

Strains: 1, YIM 93174^T; 2, *B. humi* DSM 16318^T; 3, *B. alkalitelluris* DSM 16976^T; 4, *B. litoralis* DSM 16303^T. +, Positive; −, negative; W, weakly positive; ND, no data available. Data were obtained in this study unless indicated. All strains are Gram-positive, motile rods. All strains tested negative for hydrolysis of starch, chitin and cellulose (this study). In the API ZYM system (this study), all strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase and negative for α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. In the API 50CHB system (this study), acids were not produced by any of the strains from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, arbutin, inulin, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, potassium gluconate or potassium 2-ketogluconate.

Characteristic	1	2	3	4
Colony colour*	PT	WH[a]†	CR[b]	YW[c]
Spore shape‡	S	E/S[a]	E[b]	E[c]
Spore position§	T	ST/T[a]	T[b]	C/ST/T[c]
Temperature for growth (°C)				
Range	15–45	20–40[a]	15–40[b]	4–45[c]
Optimum	28–37	30[a]	30[b]	37[c]
pH for growth				
Range	6.0–8.0	6.0–9.0[a]	7.0–11.0[b]	⩾5.5[c]
Optimum	7.0	ND	9.0–9.5[b]	7.5[c]
NaCl concentration for growth (%, w/v)				
Range	0–10	ND	0–4[b]	0–10[c]
Optimum	0–2	7[a]	0–1[b]	2–3[c]
Anaerobic growth	−	W[a]	+[b]	−[c]
Nitrate reduction	−	+	−	−
Oxidase	−	+	+	−
Catalase	+	+	−	+
Hydrolysis of:				
Aesculin	−	+	+	+
Casein	−	−	−	+
Dextrin	+	−	−	+
Tween 20	+	+	−	−
Tween 40	+	−	−	+
Tween 60	+	+	−	+
Urea	+	+	−	−
Enzymic activity (API ZYM)				
Acid phosphatase	−	+	−	+
α-Chymotrypsin	−	+	+	+
Cystine arylamidase	−	+	+	−
β-Galactosidase	−	−	+	−
α-Glucosidase	+	−	−	−
β-Glucosidase	−	−	+	+
β-Glucuronidase	−	+	+	−
Leucine arylamidase	+	+	+	−
Lipase (C14)	+	−	−	−
Trypsin	−	+	+	+
Valine arylamidase	−	+	+	−
Acid production from (API 50CH):				
N-Acetylglucosamine	−	−	−	+
Aesculin	−	−	+	+
Amygdalin	−	−	−	+
L-Arabinose	−	−	+	+
Cellobiose	−	−	+	+
D-Fructose	+	−	−	+
Galactose	−	−	−	+
Glucose	−	−	−	+
Glycogen	+	−	−	+
Glycerol	+	−	−	+

Table 1. cont.

Characteristic	1	2	3	4
D-Mannitol	−	−	+	+
Maltose	−	−	+	+
Potassium 5-ketogluconate	+	+	+	−
Raffinose	−	−	−	+
Salicin	−	−	+	+
Starch	+	−	−	+
Sucrose	+	−	+	+
Trehalose	−	−	+	+
Turanose	+	−	−	+
D-Xylose	+	−	+	+
DNA G+C content (mol%)	36.9	37.5[a]	37.9[b]	35.2[c]

*CR, Creamy; PT, pale tangerine; WH, whitish; YW, yellowish white.
†Data taken from: *a*, Heyrman *et al.* (2005); *b*, Lee *et al.* (2008); *c*, Yoon & Oh (2005).
‡E, Ellipsoidal; S, spherical.
§C, Central or paracentral; ST, subterminal; T, terminal.

sulfonamide (1.25) and tetracycline (30). The cell-wall peptidoglycan is of the A1γ type, containing *m*-Dpm as the diagnostic diamino acid. The predominant isoprenoid quinone is MK-7. The polar lipid pattern consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and two unknown phospholipids. The major cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The G+C content of the DNA of the type strain is 36.9 mol%.

The type strain is YIM 93174^T (=DSM 22388^T =KCTC 13210^T =CCTCC AA 208068^T), isolated from a salt field in Korea.

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