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

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A Gram-stain-positive, ovoid to short rod-shaped, yellow-pigmented bacterium, designated strain DY66^T, was isolated from tidal-flat sediment collected from Deukryang Bay (Republic of Korea), and its taxonomic position was investigated by using a polyphasic approach. Strain DY66^T grew optimally at 30 °C and pH 8–9 and in 2 % (w/v) NaCl. The peptidoglycan type was A4 α , L-Lys–L-Ala–D-Glu, and tyvelose and glucose were the major cell-wall sugars. The predominant menaquinones were MK-10 and MK-9. Major cellular fatty acids (>10 % of total) were anteiso-C_{15:0} and iso-C_{15:0}. The polar lipids were diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, an unknown aminophospholipid and three unknown aminolipids. The DNA G+C content was 59.1 mol%. This chemotaxonomic profile supported the assignment of strain DY66^T to the genus *Zhihengliuella*. Phylogenetic analysis based on 16S rRNA gene sequences also indicated that strain DY66^T belonged to the family *Micrococcaceae* and was related to the genus *Zhihengliuella*. On the basis of the evidence presented in this study, strain DY66^T represents a novel species of the genus *Zhihengliuella*, for which the name *Zhihengliuella aestuarii* sp. nov. is proposed. The type strain is strain DY66^T (=KCTC 19557^T =JCM 16364^T).

The genus *Zhihengliuella* (family *Micrococcaceae*) was established by Zhang *et al.* (2007) and, at the time of writing, contained three species, *Zhihengliuella halotolerans* (the type species; Zhang *et al.*, 2007), *Z. alba* (Tang *et al.*, 2009) and '*Z. salsuginis*' (Chen *et al.*, 2010; this name has not been validly published). Members of the genus have been isolated from saline soil and subterranean brine samples and are Gram-positive, catalase-positive, oxidase-negative, asporogenous, short rod-shaped or coccoid actinobacteria. The diagnostic cell-wall sugar is tyvelose and the peptidoglycan type is A4 α . In this paper, we present the results of a polyphasic taxonomic study of an isolate from tidal flat sediment in Korea and propose a novel species of the genus *Zhihengliuella* to accommodate it.

Strain DY66^T was isolated from tidal flat sediment of Deukryang Bay (34° 42' N 127° 10' E), Republic of Korea, by using the standard dilution plating technique on marine agar (MA; Becton Dickinson), and cultivated at 20 °C for

7 days. The isolate was routinely cultured on MA and maintained at –80 °C as a suspension in marine broth (MB; Becton Dickinson) containing glycerol (20 %, v/v).

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org>; Chun *et al.*, 2007). Related sequences and the novel sequence were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) and the alignment was refined using PHYDIT (<http://plaza.snu.ac.kr/~jchun/phydit/>; Chun, 1995). Phylogenetic analysis was performed by using the computer packages PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred using maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms. Distance matrices for the neighbour-joining method were generated according to the model of Jukes & Cantor (1969). The robustness of the topology of the phylogenetic trees was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DY66^T is EU939716.

Six supplementary figures are available with the online version of this paper.

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that strain DY66^T was closely related to the genera *Arthrobacter*, *Citricoccus*, *Micrococcus* and *Zhihengliuella*. The newly determined sequence was then aligned manually against representatives of the family *Micrococcaceae*. Strain DY66^T showed the highest 16S rRNA gene sequence similarity to *Arthrobacter pascens* DSM 20545^T (97.0 % sequence similarity) followed by *Micrococcus terreus* V3M1^T, *Arthrobacter woluwensis* DSM 10495^T and *Micrococcus yunnanensis* YIM 65004^T (each 96.9 %), *Arthrobacter globiformis* DSM 20124^T (96.8 %), *Zhihengliuella halotolerans* YIM 70185^T, *Micrococcus luteus* NCTC 2665^T, *Arthrobacter phenanthrenivorans* Sphe3^T and *Micrococcus antarcticus* T2^T (each 96.7 %) and *Zhihengliuella alba* YIM 90734^T, *Arthrobacter crystallopoietes* DSM 20117^T, *Citricoccus zhacaiensis* FS24^T, *Micrococcus lylae* DSM 20315^T and *Micrococcus endophyticus* YIM 56238^T (each 96.6 %); the sequence similarity to other members of the genera *Arthrobacter*, *Citricoccus*, *Micrococcus* and *Zhihengliuella* was below 96.6 %. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DY66^T belonged to the genus *Zhihengliuella* (Fig. 1). The tree topologies inferred from the neighbour-joining, maximum-likelihood and

maximum-parsimony algorithms were essentially the same (Fig. 1 and Supplementary Figs S1 and S2, available in IJSEM Online).

Growth on various standard bacteriological media was tested by using nutrient agar (NA), tryptic soy agar (TSA) and R2A agar (all from Becton Dickinson) according to the manufacturer's instructions. Cells of strain DY66^T grown on TSA containing 2 % NaCl at 30 °C for 2 days were used for physiological and biochemical tests. The Gram reaction of cells grown on TSA containing 2 % NaCl at 30 °C for 2 days was tested by using the bioMérieux Gram stain kit according to the manufacturer's instructions. The cell life cycle was examined using cultures grown on TSA and in tryptic soy broth (TSB; Becton Dickinson), both containing 2 % NaCl, for up to 2 weeks. Cell morphology was observed using a differential interference microscope (BX50; Olympus) and scanning electron microscope (S-4800; Hitachi). Endospore formation was observed with a phase-contrast microscope (TMS-F; Nikon). Motility was examined by observing cells grown in wet mounts using a phase-contrast microscope. Growth at 0–10 % (w/v) NaCl (in increments of 1.0 %) was investigated in TSB prepared according to the formula of the Becton Dickinson medium

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain DY66^T and closely related recognized species. Percentage bootstrap values (>50 %, 1000 resamplings) are given at branch points; asterisks indicate that the corresponding nodes (groupings) were also recovered in maximum-parsimony and maximum-likelihood trees. Bar, 0.01 substitutions per nucleotide position.

except that NaCl was omitted. The pH range for growth was determined in TSB containing 2% NaCl that was adjusted to pH 4–11 (in increments of 1 pH unit) by the addition of HCl or NaOH. The temperature optimum and range for growth were tested on TSA containing 2% NaCl at 4–50 °C. Anaerobic growth was tested on TSA in a jar containing an AnaeroPack-Anaero (Mitsubishi Gas Chemical), which works as an O₂ absorber and CO₂ generator, for up to 10 days. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956), respectively. Nitrate reduction was tested on TSB containing 0.2% KNO₃ (Skerman, 1967). H₂S production was determined on Kligler iron agar (Becton Dickinson). Hydrolysis of CM-cellulose (1%, w/v), casein (2% skimmed milk, w/v), egg yolk (10%, w/v), starch (0.2%, w/v), Tweens 20 and 80 (both 1%, w/v), L-tyrosine (0.5%, w/v) and xylan (1%, w/v) was tested as described by Smibert & Krieg (1994). DNase activity was determined with DNase test agar (Becton Dickinson). Other biochemical tests and enzymic activities were performed using API 20NE and API ZYM kits (bioMérieux) and the GP2 MicroPlate (Biolog) prepared according to the manufacturers' instructions. Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 5 days of incubation at 30 °C on TSA, the results were interpreted according to the guidelines set forth by the CLSI (2003).

Cells of strain DY66^T were Gram-stain-positive, non-spore-forming, non-motile and strictly aerobic. Cells were ovoid to short rod-shaped, approximately 0.7–0.9 × 0.8–1.1 µm after 3 days at 30 °C on TSA (Supplementary Fig. S3). A rod–coccus life cycle was not displayed. Detailed results of physiological and biochemical analyses are given in Table 1 and the species description. It is evident from Table 1 that there are several phenotypic characters that readily separate strain DY66^T from phylogenetically related species.

Cellular fatty acids were prepared (in duplicate) from strain DY66^T grown on TSA containing 2% NaCl for 2 days at 30 °C and analysed as methyl esters by GLC according to the instructions of the Microbial Identification System (MIDI). Quantitative analysis of amino acids was performed after derivatization by GC and GC/MS (320-MS Quadrupole; Varian) according to MacKenzie (1987). Determination of the peptidoglycan structure of strain DY66^T was carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on cellulose was applied instead of paper chromatography. The cell-wall acyl type was determined by the method of Uchida *et al.* (1999). Cell-wall sugars were prepared as described by Stanek & Roberts (1974) and analysed by TLC (silica gel 60 F₂₅₄; Merck). Seven standard sugars (0.1% w/v; Sigma) and cell-wall hydrolysates of *Z. halotolerans* YIM 70185^T and *Z. alba* YIM 90734^T were used as references. Polar lipids were analysed by using

Table 1. Differential phenotypic characteristics of strain DY66^T and related type strains

Strains: 1, DY66^T; 2, *Z. halotolerans* YIM 70185^T; 3, *Z. alba* YIM 90734^T; 'Z. salsuginis' JSM 071043 (proposed type strain). Data were obtained in this study unless indicated otherwise. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Colony colour on TSA with 2% NaCl	Yellow	Cream	Cream	Cream
NaCl concentration for growth (% w/v)				
Range	0–7	0–25	0–15	0.5–20
Optimum	2	10	5	5–10
Hydrolysis of:				
Casein	+	–	–	+
Starch	–	+	–	+
Tween 20	+	+	–	+
Tween 80	–	+	+	+
Aesculin	–	+	+	–
Gelatin	–	+	+	+
Utilization of (Biolog GP2):				
Acetic acid	–	+	–	+
N-Acetyl-D-galactosamine	–	–	+	–
D-Alanine	–	+	–	+
L-Alanine	+	–	–	+
L-Alanyl glycine	–	+	–	–
L-Arabinose	–	+	–	–
D-Arabitol	–	+	+	–
L-Asparagine	+	–	+	–
2,3-Butanediol	–	+	–	–
Cellobiose	–	–	+	+
β-Cyclodextrin	–	+	–	–
2'-Deoxyadenosine	–	–	+	–
D-Fructose 6-phosphate	–	+	–	–
Gentiobiose	–	+	–	+
α-D-Glucose 1-phosphate	–	+	–	–
D-Glucose 6-phosphate	–	+	–	–
L-Glutamic acid	+	+	–	–
DL-α-Glycerol phosphate	–	+	–	–
α-Hydroxybutyric acid	–	+	–	+
β-Hydroxybutyric acid	–	+	–	–
γ-Hydroxybutyric acid	–	+	–	+
Inosine	–	+	–	–
myo-Inositol	–	+	+	–
α-Ketoglutaric acid	–	+	–	–
α-Ketovaleric acid	–	+	+	–
Lactose	–	–	+	–
D-Malic acid	–	+	–	–
L-Malic acid	+	+	–	–
Maltose	–	+	+	–
Maltotriose	–	+	–	+
Mannan	–	+	+	–
Melibiose	–	+	–	+
Methyl β-D-galactoside	–	+	–	–
Methyl α-D-glucoside	–	+	+	–
Methyl β-D-glucoside	–	+	–	–
Methyl α-D-mannoside	–	+	–	–

Table 1. cont.

Characteristic	1	2	3	4
Palatinose	–	+	+	–
L-Pyroglutamic acid	+	–	–	+
Pyruvic acid	–	+	–	+
Pyruvic acid methyl ester	–	+	–	–
D-Psicose	–	+	+	+
Salicin	–	+	–	+
Sedoheptulosan	–	+	–	+
L-Serine	+	–	+	–
D-Sorbitol	–	+	–	+
Stachyose	–	+	–	–
Succinamic acid	–	+	–	–
Sucrose	–	+	+	–
D-Tagatose	–	+	+	–
Thymidine	–	+	+	–
Thymidine 5'-monophosphate	–	+	–	–
Trehalose	–	+	+	–
Turanose	–	+	–	–
Tween 40	–	+	+	–
Tween 80	–	+	+	–
Uridine	–	+	–	+
Uridine 5'-monophosphate	–	+	–	–
Enzyme activity (API ZYM)				
Acid phosphatase	–	–	+	–
Alkaline phosphatase	–	+	–	+
α -Chymotrypsin	–	–	+	+
Cystine arylamidase	–	–	+	–
Esterase lipase (C8)	–	+	+	+
α -Galactosidase	–	–	+	–
β -Glucosidase	–	–	+	+
Lipase (C14)	–	+	–	–
Leucine arylamidase	+	–	+	+
Valine arylamidase	–	–	+	–
Trypsin	w	–	+	+
Naphthol-AS-BI-phosphohydrolase	w	–	+	–
Esterase (C4)	–	+	+	+
Cell-wall sugars*	Tyv, Glc	Tyv, Glc ^{a†}	Tyv, Man ^b	Tyv, Man ^c
DNA G+C content (mol%)	59.1	70.3 ^a	66.5 ^b	67.8 ^c

*Glc, Glucose; Man, mannose; Tyv, tyvelose.

†Data taken from: a, Zhang *et al.* (2007); b, Tang *et al.* (2009); c, Chen *et al.* (2010).

standard procedures (Minnikin *et al.*, 1984). Extracted lipids were separated by two-dimensional TLC and identified by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Respiratory quinones were extracted (from 300 mg freeze-dried cells) and purified according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described previously (Kroppenstedt, 1985). For determination of the DNA G+C content, DNA was prepared in

duplicate and the G+C content was determined by the thermal denaturation method of Marmur & Doty (1962) and calculated using the formula described by Mandel *et al.* (1970).

The fatty acid profile of strain DY66^T is shown in Table 2. The predominant fatty acids were anteiso-C_{15:0} (59.5 %) and iso-C_{15:0} (15.8 %), similar to *Z. halotolerans*. The absence of anteiso-C_{17:0} differentiates strain DY66^T from *Z. halotolerans*, *Z. alba* and '*Z. salsuginis*' (Table 2).

The major menaquinone was MK-10 (71.2 %), and a smaller amount of MK-9 (28.8 %) was present, as for all *Zhihengliuella* species. The absence of MK-8 and MK-8(H₂) as major menaquinones differentiates strain DY66^T from *Micrococcus* species. The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan contained the amino acids lysine, glutamic acid and alanine in a molar ratio of about 1.0:1.8:2.1. The peptidoglycan partial hydrolysate (4 M HCl, 0.75 h, 100 °C) contained (in addition to the amino acids) the peptides L-Ala→D-Glu, L-Lys←L-Ala, L-Lys→D-Ala and L-Ala→L-Lys→D-Ala, as for all *Zhihengliuella* species. Dinitrophenylation according to Schleifer (1985) revealed that glutamic acid represents the N terminus of the interpeptide bridge. Dinitrophenylated alanine was also detected, in smaller amounts. From these data, it was concluded that strain DY66^T shows the peptidoglycan type

Table 2. Cellular fatty acid profiles of strain DY66^T and type strains of *Zhihengliuella* species

Strains: 1, DY66^T; 2, *Z. halotolerans* YIM 70185^T; 3, *Z. alba* YIM 90734^T; 4, '*Z. salsuginis*' JSM 071043. Cells of all strains were harvested after cultivation at 30 °C on TSA containing 2 % NaCl for 2 days. Values are mean percentages of total fatty acids from two determinations and were obtained in this study; fatty acids that represented <0.5 % in all four strains have been omitted. –, Not detected.

Fatty acid	1	2	3	4
Straight-chain				
C _{14:0}	1.4	1.1	1.0	1.3
C _{16:0}	6.9	5.2	5.8	7.1
C _{18:0}	5.6	1.2	2.6	2.8
Branched				
iso-C _{14:0}	3.4	0.6	0.9	0.6
iso-C _{15:0}	15.8	8.3	5.1	10.9
iso-C _{16:0}	2.9	5.2	5.6	4.3
iso-C _{17:0}	–	1.2	0.8	1.2
anteiso-C _{13:0}	0.6	–	0.1	–
anteiso-C _{15:0}	59.5	61.2	63.0	57.7
anteiso-C _{17:0}	0.5	14.3	13.5	11.5
Unsaturated				
iso-C _{16:1} H	0.5	–	–	–
C _{18:1ω7c}	0.9	–	0.4	0.3
C _{18:1ω9c}	0.8	–	0.4	0.3
Hydroxy				
C _{18:1} 2-OH	–	1.7	0.5	1.7

A4 α (L-Lys $\leftarrow$ L-Ala $\leftarrow$ D-Glu) (Supplementary Fig. S4). The major cell-wall sugars were tyvelose and glucose; a minor amount of galactose was also detected, similar to *Zhihengliuella* species (Supplementary Fig. S5). Tyvelose is the characteristic sugar of the genus *Zhihengliuella*, and is not found in strains of the genera *Kocuria*, *Nesterenkonia*, *Arthrobacter* or *Micrococcus* of the family *Micrococcineae* (Zhang *et al.*, 2007; Tang *et al.*, 2009). The polar lipid pattern was characterized by the presence of diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, an unknown aminophospholipid and three unknown aminolipids (Supplementary Fig. S6). The DNA G + C content of strain DY66^T was 59.1 mol%.

Chemotaxonomic studies indicated that strain DY66^T is affiliated with the genus *Zhihengliuella*. A number of phenotypic characters clearly distinguish strain DY66^T from phylogenetically related *Zhihengliuella* species (Table 1). Therefore, strain DY66^T represents a novel species within the genus *Zhihengliuella*, for which the name *Zhihengliuella aestuarii* sp. nov. is proposed.

Description of *Zhihengliuella aestuarii* sp. nov.

Zhihengliuella aestuarii (aes.tu.a'ri.i. L. gen. n. *aestuarii* of a tidal flat, from where the type strain was isolated).

Cells are strictly aerobic, Gram-stain-positive, non-motile, non-spore-forming, ovoids to short rods, approximately 0.7–0.9 $\times$ 0.8–1.1 μ m after 3 days at 30 °C on TSA. No rod-coccus life cycle. Catalase-positive and oxidase-negative. Colonies grown on TSA plates for 3 days at 30 °C are yellow, circular, opaque and convex with entire margins, approximately 1 mm in diameter. Growth occurs on TSA at 4–37 °C; optimal temperature for growth is 30 °C. The pH range for growth is 6–10; optimum pH 8–9. Grows in the presence of 0–7 % (w/v) NaCl; no growth in the presence of 8 % NaCl. Does not produce H₂S. Casein is hydrolysed, but CM-cellulose, DNA, egg yolk, Tweens 20 and 80, starch, L-tyrosine and xylan are not. In the API 20NE system (bioMérieux), gives positive results for assimilation of D-glucose and maltose and negative results for nitrate reduction, indole production, D-glucose fermentation, aesculin hydrolysis (β -glucosidase), arginine dihydrolase, β -galactosidase, gelatin hydrolysis, urease and assimilation of N-acetylglucosamine, adipic acid, L-arabinose, capric acid, malic acid, D-mannitol, D-mannose, phenylacetic acid, potassium gluconate and trisodium citrate. In the API ZYM gallery, α -glucosidase and leucine arylamidase activities are present and naphthol-AS-BI-phosphohydrolase and trypsin activities are weakly positive; N-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, α -chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α -fucosidase, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, lipase (C14), α -mannosidase and valine arylamidase activities are absent. The following carbon sources are utilized (positive with the Biolog GP2 system): L-alanine, L-asparagine, dextrin, D-fructose, α -D-glucose, L-glutamic acid, L-malic acid, D-mannose, D-ribose (weak),

L-pyrogutamic acid (weak), L-serine and D-sorbitol (weak). The remaining substrates of the Biolog GN2 system are not utilized. Cells are sensitive to (μ g per disc, unless otherwise indicated): ampicillin (10), penicillin (10 IU) and vancomycin (30) but resistant to amikacin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), polymyxin B (300 IU), streptomycin (10) and tetracycline (30). Major fatty acids are anteiso-C_{15:0} and iso-C_{15:0}. Peptidoglycan type is A4 α , L-Lys $\leftarrow$ L-Ala $\leftarrow$ D-Glu. The glycan moiety of the peptidoglycan contains acetyl residues. Major cell-wall sugars are tyvelose and glucose; a minor amount of galactose is also detected. The predominant isoprenoid quinone is MK-10; a minor amount of MK-9 is also detected. The polar lipids are diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, an unknown aminophospholipid and three unknown aminolipids. The DNA G + C content of the type strain is 59.1 mol%.

The type strain is strain DY66^T (=KCTC 19557^T =JCM 16364^T), isolated from tidal flat sediment collected from Deukryang Bay, Republic of Korea.

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