Zooshikella ganghwensis gen. nov., sp. nov., isolated from tidal flat sediments

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Two red pigment-producing bacterial strains with a metallic green sheen were isolated from a sediment sample of getbol, the Korean tidal flat. Phylogenetic analysis based on 16S rDNA sequences showed that these isolates represent a phyletic lineage within the γ -*Proteobacteria* that is distantly related to the genus *Hahella*. No bacterial species with validly published names showed $\geq 92\%$ 16S rRNA similarity with the getbol isolates. The strains were Gram-negative, chemo-organotrophic, aerobic and required NaCl (1–7%) for growth. They produced pigments with maximum absorption at 540 nm, which indicated the presence of prodigiosin, a well-known red pigment previously detected in *Serratia marcescens*. The major isoprenoid quinone was ubiquinone-9. The predominant cellular fatty acids were saturated and monounsaturated straight-chain fatty acids. The DNA G + C contents ranged from 40 to 42 mol%. The combination of physiological, biochemical and chemotaxonomic data clearly separated the test strains from other phylogenetically related genera in the γ -*Proteobacteria*. On the basis of polyphasic evidence from this study, it is proposed that the two getbol isolates should be classified in a novel genus, *Zooshikella* gen. nov., as *Zooshikella ganghwensis* sp. nov.

The Korean tidal flat, called getbol, is the neutral zone that connects land and sea; it is largely found along the west coast of the Korean peninsula. It has been well-known for its vital biological functions, such as bioremediation of pollutants, fishery products, flood control, spawning and nursery grounds for marine animals, which may result from microbial activities and diversity (Korea Ocean Research & Development Institute, 1998). However, members of microbial communities in getbol are largely unknown, as they have never been the subject of systematic taxonomic investigation. Recently, we have initiated a study that involves the isolation and identification of members of the aerobic bacterial community from getbol. In this study, we present the taxonomic properties of two isolates, for which the name *Zooshikella ganghwensis* sp. nov. is proposed.

Isolation

Two aerobic, halophilic bacterial strains were isolated from a sediment sample collected from the getbol of Ganghwa

Island in Korea (37° 35' 31.9" N, 126° 27' 24.5" E). The sample was diluted with sterilized artificial sea water (ASW; Lyman & Fleming, 1940), spread onto a plate containing marine agar 2216 (MA; Difco) and incubated at 25 °C for 3 weeks. The isolates were routinely cultured on MA and maintained as glycerol suspensions (20%, w/v) at –80 °C.

Molecular systematics

16S rDNA was enzymically amplified from a single colony. Primers, PCR conditions and sequencing were performed as described elsewhere (Chun & Goodfellow, 1995). The 16S rDNA sequences of strains JC2044^T and JC2045 were manually aligned with representative sequences of the γ -*Proteobacteria* obtained from GenBank. Phylogenetic trees were inferred by using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The alignment and phylogenetic analyses were carried out using the PHYDIT (available at <http://plaza.snu.ac.kr/~jchun/phydit/>)

Published online ahead of print on 29 November 2002 as DOI 10.1099/ijs.0.02521-0.

The GenBank accession numbers for the 16S rDNA sequences of strains JC2044^T and JC2045 are AY130994 and AY130995, respectively.

and PAUP 4.0 (Swofford, 1998) programs, as described previously (Chun *et al.*, 2000).

Nearly complete 16S rDNA sequences of strains JC2044^T and JC2045 were obtained (1441 and 1448 bp, respectively). Preliminary sequence comparison against 16S rDNA sequences in GenBank indicated that our isolates belonged to the γ -Proteobacteria. On the basis of 16S rDNA similarity, our isolates showed no apparent relationship with other bacteria: no validly published bacterial species showed 92 % or higher 16S rRNA sequence similarity with the test strains. However, the sequence of an uncultured marine bacterium (GenBank no. AJ315452) showed 99 % 16S rDNA similarity with our isolates. It is noteworthy that this sequence was derived from a sample of sea water from Dayawan Bay near Hong Kong. The result may indicate that taxa related to our isolates may be widespread in the marine environment that stretches from the Yellow Sea to the South China Sea.

The closest cultured bacterial relatives were *Marinobacter aquaeolei* DSM 11845^T (91 %), *Marinobacter hydrocarbonoclasticus* ATCC 27132 (91 %), *Microbulbifer hydrolyticus* DSM 11525^T (91 %), *Hahella chejuensis* KCTC 2396^T (90 %), *Oceanospirillum multiglobuliferum* NBRC 13614^T (89 %) and *Oceanospirillum linum* ATCC 11336^T (89 %). This distant relationship between our isolates and cultured bacteria was also evident in the phylogenetic tree, where the two test strains, together with the sequence of an uncultured marine bacterium (GenBank number AJ315452), formed a monophyletic clade with a bootstrap value of 100 % that was supported by all tree-making methods employed in this study (Fig. 1). *Hahella chejuensis* KCTC 2396^T was recovered as a sister taxon to the clade that contained our isolates, although the relationship was not found in the maximum-likelihood and parsimony analyses. It is evident from phylogenetic analysis that our isolates represent a novel phyletic lineage in the γ -Proteobacteria.

The 16S rDNA similarity value between strains JC2044^T and JC2045 was 99.7 %, corresponding to four nucleotide differences. Genomic relatedness between these isolates was examined further by using slot-blot DNA–DNA hybridization (Chun *et al.*, 1998). The two isolates shared a high DNA–DNA relatedness value of 93 %, which is above the 70 % threshold for delineating bacterial species (Wayne *et al.*, 1987). It is clear from this DNA–DNA pairing study that the strains belong to the same genomic species.

Morphology and physiological properties

Cellular morphologies were examined after growth on MA at 30 °C for 2 days by scanning and transmission electron microscopy. The motility of young cells was determined by phase-contrast microscopy. Cells were Gram-negative, slightly curved rods, 0.7–0.9 μ m in width and 1.5–2.5 μ m in length and actively motile with a single polar flagellum (Fig. 2). Spore formation was not observed under any of the growth conditions described in this study. The colonies

Fig. 1. Neighbour-joining tree based on nearly complete 16S rDNA sequences, showing relationships between strains JC2044^T and JC2045 and members of the γ -Proteobacteria. Numbers at the nodes are levels of bootstrap support (%), based on neighbour-joining analyses of 1000 resampled datasets. Asterisks indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Helicobacter pylori* ATCC 43504^T (U01330) was used as an outgroup. Bar, 0.1 nucleotide substitutions per position.

were circular, convex, entire, glistening, opaque and viscid on agar plates. When grown on MA at 30 °C, they were approximately 1 mm in diameter after 36 h and 1–2 mm after 3 days, and reached their maximum diameter of 4–5 mm after 1 week. The test strains produced non-diffusible, water-insoluble intracellular pigments, regardless of the presence of light. The colonial colours of strains JC2044^T and JC2045 were yellowish-red and red, respectively. Both strains produced a metallic green sheen on plates of MA, CSY-3 (Sawabe *et al.*, 1998), medium B

Fig. 2. Scanning electron micrograph of strain JC2044^T. Cells were grown on MA at 30 °C for 2 days. Bar, 1 μ m.

(Ivanova *et al.*, 1996), yeast extract agar (YEA; Baumann *et al.*, 1972) and YTSS agar (Gonzalez *et al.*, 1997). However, colonies grown on basal medium (BM; Baumann *et al.*, 1972) agar showed little pigmentation and no metallic sheen. When grown on MA at 30 °C, the strains showed translucent colonies at early stages of growth (12–18 h), then changed to a faint reddish hue (24 h) and finally became deep red with a metallic sheen (24–30 h). A lower extent of metallic sheen was produced at 35 °C than at 15–30 °C. When grown in liquid medium, the test strains produced abundant red pigment but no metallic sheen.

A distinctive feature of our isolates was their ability to produce a large amount of red pigment. The pigment showed characteristics similar to prodigiosin, as it is insoluble in water and has a red colour with a metallic green sheen. Prodigiosin has been reported to be deposited in the cell envelope and not released into the medium; in addition, in its amorphous state it has an appearance of red platelets with a green metallic sheen (Margalith, 1992), and a maximum absorption of 535–540 nm (Allen, 1967). *Serratia marcescens* is well-known as a producer of prodigiosin. The red cellular pigments of our isolates were extracted with absolute ethanol from freeze-dried biomass and analysed spectrophotometrically (Gauthier & Breittmayer, 1992). The absorption spectra of crude extracts were determined in the range 200–800 nm using a spectrophotometer (Ultrospec 2000, Amersham Biosciences). The absorption spectra of pigments from our isolates were nearly identical to that of prodigiosin isolated from *S. marcescens* ATCC 27117, with a maximum peak at 540 nm. However, the next largest peaks were slightly different in all spectra (471–473 nm for *S. marcescens*, 495–499 nm for strain JC2044^T and 491–496 nm for strain JC2045).

To examine bacterial growth, the isolates were inoculated onto several bacteriological growth media. Abundant growth was observed on medium B and YTSS, and the best growth was observed on CSY-3, YEA and MA. The isolates grew weakly on BM, BM with 0.01 % yeast extract (Baumann *et al.*, 1972) and the basal medium for *H. chejuensis* (Lee *et al.*, 2001), and failed to grow on Luria–Bertani (supplemented with ASW), nutrient (supplemented with ASW), PYSE (Yumoto *et al.*, 1998) and tryptic soy (supplemented with ASW) agar plates. After various media were evaluated, BM and BM with 0.01 % yeast extract were chosen as the basal media for the carbon source utilization test.

The two getbol isolates showed little growth under anaerobic conditions produced by the GasPak system (BBL). The pH range for growth was 5–8 and optimal growth was observed at pH 7 in medium B broth. The test strains had an absolute requirement for Na⁺ in the range 1–7 % (w/v) NaCl, and the optimum concentration was 3–4 % (w/v) in medium B broth. The temperature range for growth was 15–45 °C and the optimum temperature was 35 °C on MA, YEA and CSY-3 agar plates and in

medium B broth. Extended incubation (up to 1 month) was required at 15 °C.

Standard physiological and biochemical tests were performed as described previously (Baumann *et al.*, 1972; Koneman *et al.*, 1979; Smibert & Krieg, 1994). Decarboxylation of amino acids (arginine, lysine and ornithine) was determined after 4 days, using decarboxylase medium base (Ewing *et al.*, 1960) supplemented with 2.5 % (w/v) NaCl. Production of H₂S was detected in triple-sugar iron agar supplemented with 2.5 % (w/v) NaCl and fluorescein production was tested in fluorescence-denitrification medium supplemented with 2.5 % (w/v) NaCl. For determination of acetoin production, strains were grown in YEB broth with 1 % glucose according to Baumann *et al.* (1972). After 72 h incubation, the Voges–Proskauer test was performed. Oxidation or fermentation of glucose was examined using marine oxidation–fermentation medium (Leifson, 1963). Hydrolyses of agar, alginic acid, casein, chitin, lecithin, starch and Tween 80 were tested using MA as the basal medium. Degradation of cellulose was tested on MA containing 0.5 % CM-cellulose (Sigma) and the Congo red test (Skipper *et al.*, 1985) was performed after 7 days at 35 °C. DNase test agar (Difco) supplemented with 50 % ASW was used for the DNase assay. Accumulation of poly-β-hydroxybutyrate was determined by staining nitrogen-limited cells (grown on BM agar containing 0.1 % D-glucose and 0.01 % yeast extract) with Nile blue A, according to Ostle & Holt (1982). Utilization of various organic substrates as sole carbon sources (at a concentration of 0.1 %, w/v) was tested on synthetic solid BM and BM with 0.01 % yeast extract (Difco) agar. Additional biochemical tests were performed using the API 20NE and API ZYM kits (bioMérieux). Strips were inoculated with a heavy bacterial suspension in ASW or AUX medium supplemented with 20 ‰ sea salt (Sigma). The results of biochemical and physiological tests are summarized in Table 1. Strains JC2044^T and JC2045 had almost-identical physiological and biochemical profiles. Only six traits were different between these strains, as indicated in Table 1.

Chemotaxonomy

Isoprenoid quinones were analysed by using HPLC, according to Minnikin *et al.* (1984) and Collins (1985). The predominant isoprenoid quinone of the test strains was ubiquinone-9 (Q-9). Fatty acid methyl esters were prepared from biomass that was scraped from MA after 5 days incubation at 30 °C and analysed by GC, according to the instructions of the Microbial Identification system (MIDI). The profiles of cellular fatty acids are given in Table 2. Strains JC2044^T and JC2045 gave almost-identical patterns; the predominant fatty acids were saturated and monounsaturated.

DNA was prepared according to Wolff & Gemmill (1997) and the G+C content of the DNA was determined by HPLC of deoxyribonucleosides as described by Mesbah *et al.* (1989), using a Supelcosil LC-18-S reverse-phase

Table 1. Physiological and biochemical features of strains JC2044^T and JC2045

+, Positive reaction; −, negative reaction; w, weakly positive. Strains JC2044^T and JC2045 were positive in the tests for catalase, oxidase, arginine dihydrolysis, ornithine decarboxylation, hydrolysis of DNA, gelatin and lecithin, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase. Weakly positive reactions were observed for cystine arylamidase and naphthol-AS-BI-phosphohydrolase. Both strains showed negative reactions in the tests for lysine decarboxylation, fluorescein, β -galactosidase, poly- β -hydroxybutyrate accumulation, urease, production of acetoin, H₂S and indole, hydrolysis of aesculin, agar, alginic acid, casein, cellulose, chitin, starch and Tween 80, activities of trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase. Organic compounds utilized by both strains were as follows: D-fructose, D-glucose, D-ribose, D-trehalose, L-asparagine, L-tyrosine, malate, maltose, N-acetylglucosamine and succinate. Adenine was weakly utilized. Organic compounds utilized by neither strain were as follows: acetamide, acetate, adipate, benzoate, caprate, citrate, D-cellobiose, D-galactose, D-mannitol, D-mannose, D-raffinose, D-salicin, D-sorbitol, D-xylose, ethanol, gluconate, glycerol, glycine, inositol, inulin, lactose, L-arabinose, L-ascorbate, L-cysteine, L-lysine, L-rhamnose, PEG, phenylacetate, propan-2-ol, sucrose and thiamin. Both strains were able to produce acid fermentatively from glucose.

Characteristic	JC2044 ^T	JC2045
Nitrate reduction	w	+
API ZYM:		
Lipase (C14)	−	+
α -Glucosidase	+	w
N-Acetyl- β -glucosaminidase	−	+
Utilization of:		
L-Arginine	w	+
L-Ornithine	w	+

Table 2. Cellular fatty acid composition (%) of strains JC2044^T and JC2045

Values are percentages of total fatty acids. Tr, <0.5 %.

Fatty acid	JC2044 ^T	JC2045
C _{10:0}	2.6 ± 0.1	3.1 ± 0.1
C _{10:0} 3-OH	2.1 ± 0.1	3.2 ± 0.1
C _{12:0}	Tr	1.7 ± 0.5
C _{12:1} 3-OH	Tr	0.8 ± 0.0
C _{12:0} 3-OH	5.1 ± 0.4	5.5 ± 0.2
C _{14:0}	6.0 ± 0.1	5.4 ± 0.5
C _{16:0}	31.9 ± 0.9	29.5 ± 1.0
C _{16:1} ω 7c	37.1 ± 0.1	36.1 ± 1.5
C _{18:1} ω 7c	14.5 ± 0.5	13.2 ± 0.6
C _{18:0}	0.7 ± 0.2	0.5 ± 0.1

column (Supelco). The DNA G+C contents of strains JC2044^T and JC2045 were 42 and 40 mol%, respectively.

Taxonomic conclusions

Very low 16S rDNA similarity values (<92 %) and the formation of a distinctive phyletic lineage clearly indicate that the two getbol isolates can be assigned to a novel genus in the γ -Proteobacteria. In addition, a number of phenotypic characters can be used to separate our isolates from their phylogenetic neighbours, the genera *Hahella*, *Microbulbifer* and *Marinobacter* (Table 3). On the basis of the polyphasic evidence presented in this study, it is proposed that the two getbol isolates should be classified in a novel genus and species as *Zooshikella ganghwensis* gen. nov., sp. nov.

Description of *Zooshikella* gen. nov.

Zooshikella (Zoo.shik.el'la. N.L. fem. n. *Zooshikella* named after Zoo Shik Lee, a Korean pioneer in food microbiology).

Gram-negative, aerobic, chemo-organotrophic, halophilic bacteria. Oxidase- and catalase-positive. Cells are rod-shaped, slightly curved and motile by a single polar flagellum. NaCl is required (1–7 %, w/v) for growth. Major isoprenoid quinone is Q-9. Predominant cellular fatty acids are saturated and monounsaturated straight-chain fatty acids. DNA G+C content is 40–42 mol%. Phylogenetically, this genus is affiliated to the γ -Proteobacteria. The type species is *Zooshikella ganghwensis*.

Description of *Zooshikella ganghwensis* sp. nov.

Zooshikella ganghwensis (gang.hwen'sis. N.L. fem. adj. *ganghwensis* named after Ganghwa Island in Korea, the geographical origin of the type strain of the species).

In addition to the characteristics that define the genus, the species has the characteristics described below. Optimal growth is observed at 35 °C, pH 7 and 3–4 % (w/v) NaCl. Grows on MA, CSY-3, medium B, YEA and YTSS agar as circular, convex, entire, glistening, opaque and viscid colonies that are yellowish-red or red, approximately 1 mm in diameter after 36 h on MA at 30 °C and reach the maximum diameter of 4–5 mm after 7 days. Cells are 0.7–0.9 μ m in width and 1.5–2.5 μ m in length. Detailed physiological and biochemical characteristics are given in Table 1. Large amounts of red pigment (maximum absorption at 540 nm) with a metallic green sheen are produced on agar medium. Major fatty acids are C_{16:0}, C_{16:1} ω 7c and C_{18:1} ω 7c. DNA G+C content is 40–42 mol%.

The type strain, JC2044^T (=IMSNU 14003^T=KCTC 12044^T=DSM 15267^T), was isolated from the sediment of getbol, the Korean tidal flat.

Table 3. Major characteristics that differentiate strains JC2044^T and JC2045 from other phylogenetically related bacteria

Taxa: 1, strains JC2044^T and JC2045; 2, *Hahella chejuensis*; 3, *Microbulbifer hydrolyticus*; 4, *Marinobacter aquaeolei*; 5, *Marinobacter hydrocarbonoclasticus*. Data are from this and earlier studies (Gauthier *et al.*, 1992; González *et al.*, 1997; Nguyen *et al.*, 1999; Lee *et al.*, 2001). ND, Not determined.

Characteristic	1	2	3	4	5
Pigmentation	Yellowish-red or red	Pinkish-red	Cream	Yellow	Pinky
Relation to oxygen	Aerobe	Facultative aerobe	Aerobe	Aerobe	Aerobe
Temperature range for growth (°C)	15–45	10–45	10–41	13–50	10–45
pH range for growth	5–8	6–10	6.5–8.5	5–10	6–9.5
Na ⁺ requirement	1–7 %	1–8 %	0.1–0.5 M	0.3–4 M	0.6–1.5 M
Arginine dihydrolase	+	–	ND	+	–
Ornithine decarboxylase	+	–	ND	ND	–
Nitrate reduction	+	+	–	+	+
Acid production from glucose	+	+	–	–	–
Aesculin hydrolysis	–	+	ND	+	–
Urease	–	–	ND	+	–
DNase	+	ND	ND	ND	–
Gelatin hydrolysis	+	+	+	+	–
Amylase	–	ND	+	ND	–
Tween 80 hydrolysis	–	ND	+	ND	+
Utilization of:					
Acetate	–	ND	+	+	+
Citrate	–	–	–	+	+
D-Cellobiose	–	+	+	–	ND
D-Fructose	+	+	–	–	ND
D-Glucose	+	+	+	–	ND
D-Mannitol	–	+	ND	–	ND
D-Mannose	–	+	–	–	ND
D-Ribose	+	–	ND	ND	–
D-Sorbitol	–	+	ND	ND	–
D-Xylose	–	–	+	ND	ND
Glycerol	–	+	–	–	ND
Inositol	–	+	–	–	–
L-Arginine	+	ND	+	–	ND
L-Ornithine	+	ND	ND	–	–
L-Tyrosine	+	ND	ND	–	ND
Malate	+	–	+	ND	ND
Maltose	+	+	ND	–	ND
N-Acetylglucosamine	+	ND	+	–	–
Sucrose	–	+	–	–	ND
Thiamin	–	ND	+	ND	ND
DNA G + C content (mol%)	40–42	55	58	56	53
Major fatty acids	C _{16:0} , C _{16:1} ω7c, C _{18:1} ω7c	C _{12:0} 3-OH, C _{16:0} , C _{16:1} ω9c, C _{18:0}	i-C _{15:0} , i-C _{17:1} ω9c	C _{16:0} , C _{16:1} ω9c, C _{18:1} ω9c	C _{16:0} , C _{18:1} ω9c

Acknowledgements

We are grateful to J. P. Euzéby for help with nomenclature. This work was supported by the Korea Research Foundation (project no. KRF-2001-041-D00207).

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