***Hahella chejuensis* gen. nov., sp. nov., an extracellular-polysaccharide-producing marine bacterium**

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A bacterial strain, designated 96CJ10356^T, which produced abundant extracellular polysaccharides and red pigment was isolated from marine sediment collected from Marado, Cheju Island, Republic of Korea. The organism is Gram-negative, aerobic, rod-shaped and motile. Growth was not observed in the absence of NaCl, and was optimal at an NaCl concentration of 2%. The strain contained oxidase and catalase, and was able to hydrolyse aesculin and gelatin. The major cellular fatty acids were saturated or monounsaturated straight-chain fatty acids. An almost complete 16S rDNA sequence of the test strain was determined. Phylogenetic analysis based on the neighbour-joining and Fitch–Margoliash methods indicated that the organism formed a distinct phyletic line within the γ *Proteobacteria*. This relationship was also supported by sequence comparison, as no valid bacterial species showed more than 90% sequence homology with the isolate. It is clear from polyphasic evidence that the isolate merits the status of genus in the γ subclass of the *Proteobacteria*, and the name *Hahella chejuensis* gen. nov., sp. nov. is proposed for the marine isolate 96CJ10356^T (= KCTC 2396^T = IMSNU 11157^T).

Keywords: *Hahella chejuensis* gen. nov., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

Microbial extracellular polysaccharides (EPSs) have been used in a wide variety of industrial applications such as emulsification, gel formation, absorption, film formation and anticancer treatment (Fu & Tseng, 1990; Irene *et al.*, 1990; Martins *et al.*, 1990; Low *et al.*, 1998). They have been found in various micro-organisms including *Alteromonas*, *Cyanothece*, *Pseudomonas*, *Vibrio* and *Zoogloea* (Ikeda *et al.*, 1982; Rodrigues & Bhosle, 1991; Matsuda & Worawattanamatekul, 1993; Philippis *et al.*, 1993; Raguenes *et al.*, 1996). Microbial EPSs have an advantage over polysaccharides from other sources, as they can be produced in large quantity using a relatively simple purification process. A bacterial strain producing a large amount of EPS was isolated from a marine sediment sample collected from Marado, Cheju

Island, Republic of Korea. Our preliminary study indicated that EPSs produced by the isolate are of high molecular mass (over 2×10^6 Da), consist of galactose, glucose, xylose and ribose, and have good potential as an emulsifying agent (Ko *et al.*, 2000). In the present study, we report the polyphasic characterization of this isolate, and propose that it belongs to a novel species, which is named *Hahella chejuensis* gen. nov., sp. nov.

METHODS

Bacterial strains. A marine sediment sample was collected from Marado, Cheju Island, Republic of Korea, at a depth of 1 m. The sample was diluted with saline (0.85% NaCl), spread onto ZoBell's medium (5 g peptone, 1 g yeast extract, 0.01 g FePO₄, 15 g agar, 250 ml distilled water, 750 ml aged seawater), and incubated at 20 °C. A bacterial strain, designated 96CJ10356^T, was isolated and shown to produce a large quantity of EPS. The strain was maintained as a glycerol suspension (20%, w/v) at –80 °C.

Morphology. The test strain was grown on basal medium (BM; 10 g tryptone, 20 g sucrose, 4 g MgSO₄, 7 g CaCl₂,

Abbreviation: EPS, extracellular polysaccharide.

The GenBank accession number for the 16S rDNA sequence of *Hahella chejuensis* KCTC 2396^T is AF195410.

0.07 g KH_2PO_4 , 0.08 g K_2HPO_4 , 5 mg FeCl_3 , 1 mg MnCl_2 , 1 mg Na_2MoO_4 , 1 mg ZnCl_2 , 10 g NaCl, 1 l distilled water) for 3 d at 37 °C. Morphology was observed by a differential interference microscope (Nikon), and Phillips model SEM 515 scanning and CM 20 transmission electron microscopes. Negative staining was performed with an aqueous solution (2%, w/v) of phosphotungstic acid adjusted to pH 7, as described previously (Robinson *et al.*, 1987).

Phenotypic tests. The basal medium (BM), incubated at 37 °C, was used for phenotypic tests, unless otherwise stated. The motility of young cells was examined using wet mounts. Growth under anaerobic conditions was determined by testing the growth of the strain on BM agar at 30 °C in an anaerobic chamber (5% CO_2 , 7% H_2 , 88% N_2 ; Forma Scientific). Catalase production was assayed by using 0.3% hydrogen peroxide with a colony taken from BM agar plates. Oxidase activity was determined by the method of Kovacs (1956). The growth temperature was tested over the range of 5–50 °C using BM broth. The requirement of NaCl for growth was tested using YMG broth (10 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract, 1 l distilled water) supplemented with concentrations of NaCl in the range of 0–20%. The pH range for growth was determined using BM broth adjusted to different pH values over the range of 3–11.

Biochemical tests were done using API 20E and 20NE strips (bioMérieux) following the manufacturer's instructions. Utilization of carbohydrates as sole carbon source was examined using modified BM that was prepared by replacing tryptone with 0.5% (w/v) NH_4Cl , and sucrose with 1% (w/v) carbohydrates. For acid production tests, bromothymol blue was added to a final concentration of 0.05 g l^{-1} .

Pigment. Strain 96CJ10356^T was grown in 100 ml BM broth at 30 °C for 7 d. Biomass was removed by centrifugation at 10000 g for 20 min, following the addition of 0.1 g diatomaceous earth (Sigma). The pigments were extracted from the supernatant using 2 vols methanol and chloroform, and analysed using a scanning UV/Visible spectrophotometer (Pharmacia Ultrospec 2000).

Cellular fatty acid analysis. Fatty acid methyl esters were prepared from biomass that was scraped from marine TSA [Tryptic Soy Agar (Difco), 50% aged seawater] incubated at 30 °C for 3 d. Cellular fatty acids of the test strain were analysed as methyl esters by GC according to the instructions of the Microbial Identification System (MIDI).

Determination of DNA base composition. DNA was prepared according to Chun & Goodfellow (1995). The mol% G + C content of the resultant DNA preparations was determined using the thermal denaturation method (Mandel & Marmur, 1968).

16S rDNA analysis. The primary structure of 16S rDNA was determined as described earlier (Chun & Goodfellow, 1995). The resultant sequence of strain 96CJ10356^T was manually aligned with representative sequences of the γ *Proteobacteria* obtained from the Ribosomal Database Project (Maidak *et al.*, 1997) and GenBank databases, using known 16S rRNA secondary structure information (Gutell, 1994). Phylogenetic trees were inferred by using the Fitch–Margoliash (Fitch & Margoliash, 1967) 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 trees were rooted using *Rhizobium leguminosarum* (GenBank accession no. D14513) as an outgroup. The

PHYLP package (Felsenstein, 1993) was used for all analyses. The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Nucleotide sequence accession numbers. The GenBank accession numbers of the published 16S rDNA sequences used in this study are Y12579 (*Alcanivorax borkumensis* DSM 11573^T), AF001375 (*Colwellia psychroerythraea* ATCC 27364^T), X67023 (*Halomonas elongata* ATCC 33173^T), X67022 (*Marinobacter hydrocarbonoclasticus* ATCC 49840^T), U58339 (*Marinobacterium georgiense* DSM 11526^T), AB006769 (*Marinospirillum minutulum* ATCC 19193^T), U58338 (*Microbulbifer hydrolyticus* DSM 11525^T), AF053734 (*Neptunomonas naphthovorans* NAG-2N-126^T), M22365 (*Oceanospirillum linum* ATCC 11336^T), X06684 (*Pseudomonas aeruginosa* DSM 50071^T) and D14555 (*Zymobacter palmae* IAM 14233^T). The sequence of *Marinomonas communis* is in RDP as Mrm. commu2.

RESULTS AND DISCUSSION

Phenotypic and chemotaxonomic characterization

The strain, isolated from marine sediment, was Gram-negative, rod-shaped, and catalase- and oxidase-positive. It grew equally well under both aerobic and anaerobic conditions. The colony was visible after 2 d incubation on BM agar at 37 °C. The colour of colonies changed from pale orange to pinkish red, and

Fig. 1. Scanning electron micrographs of strain 96CJ10356^T: (a) young (1 d) and (b) old (7 d) cultures. Bar, 10 μm .

Table 1 Phenotypic properties of isolate 96CJ10356^T

ND, Not determined; v, variable reaction.

Characteristic	Reaction of isolate
Production of:	
Acetoin	—
H ₂ S	—
Indole	—
Enzyme activity	
Arginine dihydrolase	—
Cytochrome oxidase	+
β-Galactosidase	—
Lysine decarboxylase	—
Nitrate reductase	+
Ornithine decarboxylase	—
Tryptophan deaminase	—
Hydrolysis of:	
Aesculin	+
Gelatin	+
Urea	—
Utilization as sole carbon source/acid production	
Adonitol	+ / +
L(+)Arabinose	— / —
D(+)Cellobiose	+ / ND
Citrate	— / ND
D(+)Fructose	+ / +
D(+)Galactose	— / —
D(+)Glucose	+ / +
Glycerol	+ / ND
Inositol	+ / +
D(+)Lactose	— / —
Malate	— / ND
Malonate	— / ND
D(+)Maltose	+ / +
D(−)Mannitol	+ / +
D(+)Mannose	+ / +
D(+)Melibiose	— / ND
D(+)Raffinose	— / —
L(+)Rhamnose	— / —
D(−)Ribose	— / v
D(−)Sorbitol	+ / +
Sucrose	+ / +
D(+)Trehalose	+ / +
D(+)Xylose	— / v

the colonial shape from small circular to large volcanic form, after 3 d. The cells were long rods in the young stage (1.6–9.0 µm long, 0.5–0.7 µm wide; Fig. 1a), which later became short rods in stationary cultures (1.4–1.7 µm long, 0.7–0.8 µm wide; Fig. 1b). The organism was motile by means of a single polar flagellum. Growth occurred between 20 and 45 °C. Extended incubation (up to 1 month) was required at 10 and 15 °C. The test strain grew in YMG broth with 1–8% NaCl, but not with 10%, and at pH 6–10, but not pH 5. Optimal growth was observed in BM broth

Table 2 Cellular fatty acid profile of isolate 96CJ10356^T

Fatty acid	Composition (%)
Dodecanoic acid	6.3
3-Hydroxydodecanoic acid	13.3
Tetradecanoic acid	6.8
cis-9-Hexadecen-1-ol	2.3
1-Hexadecanol	1.3
cis-7-Hexadecenoic acid	6.6
cis-9-Hexadecenoic acid/iso-2-hydroxypentadecanoic acid	19.4
Hexadecanoic acid	19.1
Octadecenoic acid	24.9

Fig. 2. Neighbour-joining tree based on almost complete 16S rDNA sequences showing relationships between strain 96CJ10356^T and members of the γ Proteobacteria. The percentage numbers at the nodes indicate the levels of bootstrap support for the branch point based on neighbour-joining analyses of 1000 resampled data sets. *Rhizobium leguminosarum* (accession no. D14513) was used as an outgroup. The scale bar indicates 10 nucleotide substitutions per 100 nucleotide positions.

with 2% NaCl at pH 7. The test organism was able to hydrolyse aesculin and gelatin. The results of biochemical and physiological tests are summarized in Table 1, and the profile of cellular fatty acids is given in Table 2. The pigment produced by strain 96CJ10356^T had maximum absorptions at 501.5 and 537 nm, and was produced regardless of light. The DNA G + C ratio of the isolate was 55 mol%.

Table 3 Phenotypic characteristics that differentiate strain 96CJ10356^T from other marine or halophilic bacteria belonging to the γ subclass of the *Proteobacteria*

1, Strain 96CJ10356^T; 2, *Alcanivorax borkumensis*; 3, *Halomonas elongata*; 4, *Marinobacter hydrocarbonoclasticus*; 5, *Marinobacterium georgiense*; 6, *Marinomonas* spp.; 7, *Marinospirillum* spp.; 8, *Microbulbifer hydrolyticus*; 9, *Neptunomonas naphthovorans*; 10, *Oceanospirillum* spp. Data from this and earlier studies (Gauthier *et al.*, 1992; Gonzalez *et al.*, 1997; Satomi *et al.*, 1998; Hedlund *et al.*, 1999). NR, Not reported; v, variable.

	1	2	3	4	5	6	7	8	9	10
Relation to oxygen	Facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic	Aerobic	Aerobic	Aerobic/microaerobic	Aerobic	Facultatively anaerobic	Aerobic
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Spirilla	Rods	Rods	Spirilla
Motility/flagella	+ /Single polar	+ /Single polar	+ /Peritrichous	+ /Polar OR –	+ /Single polar	+ /Single polar	+ /Polar tuft	–	+ /Single polar	+ /Bipolar tufts
Growth at 45 °C	+	–	NR	+	–	+	–	–	–	–
Oxidase	+	+	+	+	+	–	+	+	+	+
Arginine dihydrolase	–	–	–	–	NR	–	NR	NR	NR	NR
Citrate utilization	–	NR	NR	+	NR	NR	NR	NR	NR	NR
Aesculin hydrolysis	+	–	v	NR	NR	NR	NR	NR	NR	–
Gelatin hydrolysis	+	–	v	–	NR	v	NR	NR	–	–
Lysine decarboxylase	–	–	+	–	NR	–	NR	NR	NR	NR
Ornithine decarboxylase	–	–	+	–	NR	–	NR	NR	NR	NR
Reduction of nitrate	+	+	+	+	–	–	v	–	–	–
Urease	–	–	v	–	NR	–	NR	NR	NR	–
G + C content (mol%)	55	53–54	60–61	53	55	44–48	43–45	58	46	42–51

Phylogenetic analysis

An almost complete 16S rDNA sequence of isolate 96CJ10356^T was determined (1422 bp). Preliminary sequence comparison against the 16S rRNA sequences held in the GenBank and Ribosomal Database Project (Maidak *et al.*, 1997) databases indicated that the organism belongs to the γ subclass of the *Proteobacteria*. The sequence was manually aligned against representatives of the γ *Proteobacteria* using the secondary structure model of bacterial 16S rRNA (Gutell, 1994). On the basis of 16S rDNA similarity, our isolate showed no apparent relationship with representative γ *Proteobacteria*. The closest relatives are *Oceanospirillum linum* ATCC 11336^T (89.9% 16S rDNA similarity), *Microbulbifer hydrolyticus* DSM 11525^T (89.8%), *Marinobacter hydrocarbonoclasticus* ATCC 49840^T (89.6%), *Pseudomonas mendocina* LMG 1223^T (89.1%), *Oceanospirillum multiglobuliferum* IFO 13614^T (89.1%) and *Halomonas salina* ATCC 49509^T (89.0%); none of the valid bacterial species showed more than 90% sequence homology values. Phylogenetic analysis was carried out using 1302 unambiguously aligned nucleotide positions. It is evident in the neighbour-joining tree shown in Fig. 2 that the isolate formed an independent phyletic line in the γ *Proteobacteria*. The branching point of the isolate was not stable, as the corresponding bootstrap value was very low, i.e. 30%. A very similar tree topology was reconstructed by the Fitch–Margoliash treeing algorithm (data not shown).

Salt requirements

Halophilic micro-organisms can be conveniently grouped according to their requirements for NaCl for growth (Larsen, 1986): slightly halophilic marine bacteria can grow in the presence of 2–3% NaCl;

moderate halophiles grow over a much wider range of NaCl concentrations (5–20%); extreme halophiles are able to grow in saturated NaCl and are usually unable to grow with 12% or lower NaCl concentration. Our isolate can be classified as slightly halophilic, since it was unable to grow in the absence of NaCl and grew optimally with 2% NaCl. In recent taxonomic investigations, a number of marine bacteria that belong to the γ subclass of the *Proteobacteria* have been described. This group of bacteria was shown to be phylogenetically heterogeneous and physiologically diverse (Caumette *et al.*, 1997; Satomi *et al.*, 1998; Tardy-Jacquenod *et al.*, 1998; Yakimov *et al.*, 1998; Bhupathiraju *et al.*, 1999). The phylogenetic uniqueness of our isolate within the γ *Proteobacteria* further expands the diversity of the halophilic bacteria. The organism also showed distinct phenotypic traits that are different from other halophilic members of the γ *Proteobacteria*. The characteristics that differentiate isolate 96CJ10356^T from phylogenetically related marine or halophilic bacteria are summarized in Table 3. On the basis of phylogenetic and phenotypic evidence, we propose that isolate 96CJ10356^T be classified in the new genus *Hahella* as *Hahella chejuensis* gen. nov., sp. nov.

Description of *Hahella* gen. nov.

Hahella (Ha.hel'la. M.L. fem. n., named after Yung Chil Hah, a Korean bacteriologist who pioneered microbiological research in Korea).

Cells are Gram-negative, facultatively anaerobic, rod-shaped and motile by means of a single polar flagellum. The organism uses several carbohydrates as carbon sources and produces acid from sugars. Nitrate is reduced to nitrite. The organism is unable to grow in the absence of NaCl. Optimal growth at 2% (w/v) NaCl. Aesculin and gelatin are hydrolysed. Detailed

properties are given in Tables 1 and 2. Extracellular polysaccharides are produced. Phylogenetically, the genus belongs to the γ subclass of the *Proteobacteria*. The DNA G+C content of the type species is 55 mol%. The type and only species of the genus is *Hahella chejuensis*.

Description of *Hahella chejuensis* sp. nov.

Hahella chejuensis (che.ju.en'sis. M.L. adj. *chejuensis* pertaining to Cheju Island, Republic of Korea, geographical origin of the type strain of the species).

Gram-negative, halophilic, facultatively anaerobic rods. Colonies, incubated for 3 d on BM agar at 37 °C, are pinkish red and volcanic form. The cells are long rods in young cultures (1.6–9.0 µm long, 0.5–0.7 µm wide) and become short rods in stationary cultures (1.4–1.7 µm long, 0.7–0.8 µm wide). Grows at 10–45 °C, but not at 50 °C, in the presence of NaCl (1–8%) and at pH 6–10. Grows optimally with 2% NaCl and at pH 7. Methanol-soluble red pigments, with maximum absorptions at 501.50 and 537.00 nm, are produced. Phenotypic characteristics and the cellular fatty acid profile are given in Tables 1 and 2. The DNA G+C content of the type strain is 55 mol%. The type strain is 96CJ10356^T (= KCTC 2396^T = IMSNU 11157^T).

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