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NOTE

Aliihoeflea aestuarii gen. nov., sp. nov., a Novel Bacterium Isolated from Tidal Flat Sediment

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A novel Gram-negative and rod-shaped bacterium, designated N8^T, was isolated from tidal flat sediment. Phylogenetic analysis based on 16S rRNA gene sequences showed that N8^T strain is associated with the family *Phyllobacteriaceae*: two uncultured clones (98.4 and 99.8% 16S rRNA gene sequence similarity) and the genus *Mesorhizobium* (≤97.0%). The novel strain formed a separate clade with uncultured clones in the phylogenetic tree based on 16S rRNA gene sequences. Cellular fatty acid profiles predominately comprised $C_{18:1}$ *ω7c* and $C_{19:0}$ cyclo *ω8c*. The major isoprenoid quinone is ubiquinone-10 and genomic DNA G+C content is 53.4 mol%. The polyphasic taxonomic study indicates that the novel strain N8^T represents a novel species of the new genus in the family *Phyllobacteriaceae*, named *Aliihoeflea aestuarii*. The type strain is N8^T (= KCTC 22052^T= JCM 15118^T= DSM 19536^T).

***Keywords*:** *Aliihoeflea aestuarii* gen. nov., sp. nov., taxonomy

The name of the family *Phyllobacteriaceae* in the class *Alphaproteobacteria*, was first introduced by Mergaert and Swings (2005). At the time of writing, this family currently contains 8 genera, including the genus *Aminobacter*, *Aquamicrobium*, *Defluvibacter*, *Hoeflea*, *Mesorhizobium*, *Nitratireductor*, *Phyllobacterium*, and *Pseudaminobacter* (Euzeby, 1997). In this study, the polyphasic taxonomic analysis was used to establish the taxonomic position of strain N8^T, closely related to the family *Phyllobacteriaceae*, which was isolated from tidal flat sediment collected in Yeosu (34°47'26" N 127°34'01" E), Republic of Korea. The novel strain was obtained by growth on marine 2216 agar (MA, Difco) with repeated re-streaking to obtain a pure culture. The reference strains included *Hoeflea alexandrii* AM1V30^T, *M. huakuii* IAM 14158^T, *M. plurifarium* LMG 11892^T, obtained from KCTC, *H. marina* LMG 128^T from DSMZ, *H. phototrophica* DFL-43^T from NCIMB (National Collection of Industrial, Marine and Food Bacteria, Scotland), and *Mesorhizobium thiogangeticum* SJT^T from BCCM/LMG (Belgian Co-ordinated Collections of Micro-organisms).

A G-spin^{TM} DNA Extraction Kit (iNtRON Biotechnology) was used to extract template chromosomal DNA. Determination of 16S rRNA gene sequencing and phylogenetic analysis were performed as described previously (Roh *et al.*, 2008). Phylogenetic relationships between closely related species were determined using the MEGA4 (Tamura *et al.*, 2007) and PHYLIP software package (Felsenstein, 2005) software program. Distance matrices were determined (Kimura, 1980) and used to elaborate dendrograms by the neighbor-joining (Saitou and Nei, 1987), minimum evolution (Rzhetsky and Nei, 1992), maximum-parsimony (Kluge and Farris, 1969), and maximum-likelihood (Felsenstein, 1981) methods. To evaluate stability a bootstrap analysis was performed using a consensus tree that was based on 1,000 randomly-generated trees, except for maximum-likelihood method, which was done based on 300 replications. The strain N8^T 16S rRNA gene sequence (1,453 bp) is deposited at the NCBI website under accession number EF660756. The 16S rRNA gene sequence similarity indicated that strain N8^T is associated with the family *Phyllobacteriaceae*: the genus *Mesorhizobium* (95.1~97.0% 16S rRNA gene sequence similarity), *Phyllobacterium* (95.4~96.9%), *Hoeflea* (95.6~96.6%), and *Mycoplana* (90.6~96.5%), in the order of the highest 16S rRNA gene sequence similarity. It is clear that the 16S rRNA gene sequence similarity values alone do not permit to assign unequivocally the new strain to an established genus, since there are more than 30 species from 10 different genera (*Mesorhizobium*, *Phyllobacterium*, *Hoeflea*, *Mycoplana*, *Aminobacter*, *Shinella*, *Pseudaminobacter*, *Ochrobactrum*, *Rhizobium*, and *Ensifer*) that exhibit above 96.0% sequence similarity with the novel strain. The strain N8^T, however, had the highest 16S rRNA gene sequence similarity with two uncultured clones (98.4 and 99.8%), and also formed a separate clade with uncultured clones, next to the genus *Hoeflea*, as

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having high bootstrap value in the phylogenetic tree based on 16S rRNA gene sequences regardless of different tree-making algorithms using neighbor-joining, minimum evolution, maximum-parsimony, and maximum-likelihood method. Therefore, the 16S rRNA gene sequence similarity establishes strain N8^T as a distinct genospecies.

The G+C content was determined by a fluorimetric method using SYBR Green and a real-time PCR thermocycler (Gonzalez and Saiz-Jimenez, 2002). The G+C content of genomic DNA of the type strain was 53.4 mol%. The value of genomic DNA G+C content in the validated species belonging to the genus *Mesorhizobium* that showed the highest 16S rRNA gene sequence similarity with the novel strain, is in the range of 59∼65.1 mol% (Jarvis *et al.*, 1997; Gao *et al.*, 2004). The G+C content value of strain N8^T genomic DNA is relatively lower than the values for validated species of the genus *Mesorhizobium.*

For quantitative analysis of cellular fatty acid composition, the reference strains were grown under same conditions on TSA for *Mesorhizobium* strains and on MA plates for *Hoeflea* strains with the novel strain at 30°C for 2 days. Cells of each strains were harvested and cellular fatty acids were extracted and analyzed, as described previously (Roh *et al.*, 2008). The predominant cellular fatty acids in strain N8^T were $C_{18:1}$ ω7*c* (ca. 70%) and $C_{19:0}$ cyclo ω8*c* (ca. 10%). The value for $C_{16:0}$ of the novel strain is less than that of all reference strains, whereas the value for $C_{18:1}$ ω7*c* of the novel strain is higher than that of all reference strains, irrespective of media used. The fatty acid 16:0 *N* alcohol was only detected in the novel strain grown on TSA. The detailed fatty-acid composition of strain N8^T is shown in Table 2. As shown in Table 2, the fatty acid composition of strain N8^T showed unique patterns different from other genus group of *Mesorhizobium* and *Hoeflea.*

Quinones were characterized described by Collins (1985) and Wu *et al.* (1989). The 6 reference strains mentioned in the fatty acid analysis were characterized. Among the reference strains, *Hoeflea marina* LMG 128^T was only reported previously as having ubiquinone-10 (Q-10) as a major respiratory lipoquinone (Peix *et al.*, 2005). The quinone analysis showed that the strain N8^T and all of reference strains have also Q-10 as a major respiratory quinone, regardless of the both genera: *Mesorhizobium* and *Hoeflea.*

Cell morphology was examined using light microscopy (ECLIPSE 80*i*, Nikon) and electron microscopy. Gram staining was performed by Gram staining method (Gram, 1884). Requirements and tolerance of various NaCl concentrations were determined in broth medium identical to MB except for NaCl that was added at different concentrations. Growth at various temperatures (15, 17, 20, 25, 30, 37, 40, and 50°C) was measured on MA. Growth on R2A agar (Difco), Tryptic Soy Agar (TSA, Difco), Luria Agar (LA, Difco), and Yeast Mannitol Agar (YMA) (Bergersen, 1961) was also determined. Starch hydrolysis was performed as described by Smibert and Krieg (1994). Catalase and oxidase activity were determined by observing bubble production in a 3% (v/v) hydrogen peroxide solution and using an oxidase reagent (bioMérieux), respectively. API20NE, API ZYM test strips (bioMérieux), and Biolog GN plates with GN/GP inoculating fluid were used to assay enzyme activity and substrate utilization from sole carbon sources. The detailed species description is presented below and Table 1 shows a comparison between the characteristics of N8^T and closely related strains.

Root and stem nodule bacteria of legumes, belonging to the genus *Mesorhizobium*, *Rhizobium*, and *Bradyrhizobium*, are known to secrete copious amounts of exopolysaccharides on YMA media containing sugars, resulting in slimy

Table 1. Differentiating characteristics of *Aliihoeflea aestuarii* N8^T and the type strains of related taxa
Taxa: 1, N8^T; 2, *Hoeflea alexandrii* AM1V30^T (data from Palacios *et al.*, 2006); 3, *H. marina* LMG 128^T (Peix *et al.*, 2005); 4, *Mesorhizobium thiogangeticum* SJT^T (Ghosh and Roy, 2006); 5, *Phyllobacterium trifolii* PETP02^T (Valverde *et al.*, 2005); 6, *Nitratireductor aquibiodomus* NL21^T (Labbe *et al.*, 2004); 7, *Shinella granuli* Ch06^T (An *et al.*, 2006); 8, *Ochrobactrum pseudintermedium* ADV31^T (Teyssier *et al.*, 2007); 9, *Rhizobium daejeonense* L61^T (Quan *et al.*, 2005). +, positive; -, negative; w, weak reaction; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9
Temperature range for growth (°C)	17∼37	10∼42	4∼37	NR	4∼37	NR	4-40	25-45	NR
Maximum temperature (°C)	37	42	37	37	37	NR	40	45	41
NaCl (w/v) range for growth	0∼8.0	0∼11.8	0∼5	NR	0∼3	0∼5	1-4	NR	0-2
Tolerance of 3% (w/v) NaCl	+	+	+	-	+	+	+	NR	-
Nitrate reduction	-	-	-	+[a]	-	+	+	+	-
Urease	+	-	+	-[a]	w	-	+	-	+
β-Glucosidase (aesculin hydrolysis)	-	+	+	-[a]	w	-	+	-	+
β-Galactosidase (PNPG hydrolysis)	-	+	+	-[a]	-	-	+	-	+
Assimilation of									
Glucose	-	-	+	+	+	+	NR	+	+
Arabinose	+	-	+	+	+	+	+	+	+
Maltose	-	-	+	+	+	-	+	+	+
DNA G+C content (mol%)	53.4	59.7	53.1	59.6	56.4	57	66	54.5	60.1

[a] data from this study using API 20NE system

Table 2. Fatty acid content (%) of strain N8^T and related species in the genus *Hoeflea* and *Mesorhizobium*
Taxa: 1, strain N8^T (grown on MA); 2, *Hoeflea alexandrii* AM1V30^T (MA); 3, *H. marina* LMG 128^T (MA); 4, *H. phototrophica* DFL-43^T (MA); 5, strain N8^T (TSA); 6, *Mesorhizobium thiogangeticum* SJT^T (TSA); 7, *M. huakuii* IAM 14158 ^{T} (TSA); 8, *M. plurifarium* LMG 11892^T (TSA). All of data are from this study. Strains in column 1~4 and column 5~8 were grown on MA and TSA at 30°C for 2 days, respectively. Values shown are percentages of total fatty acids. tr, trace (less than 1.0%); -, not detected.

Fatty acid	**1**	2	3	4	**5**	6	7	8
14:0	**-**	tr	-	-	**-**	1.2	-	-
15:0 iso	**tr**	-	-	-	**-**	9.6	-	-
16:0	**2.1**	8.6	9.3	6.5	**1.6**	3.8	11.9	12.3
16:0 *N* alcohol	**-**	-	-	-	**2.4**	-	-	-
17:0	**3.2**	tr	tr	tr	**tr**	2.7		tr
17:0 iso	**tr**	-	-	-	**tr**	1.5	5.5	5.3
17:1ω8*c*	**1.5**	-	tr	tr	**-**	-	-	-
18:0	**3.6**	1.5	1.9	1.4	**3.1**	3.1	5.1	5.4
18:1ω7*c*	**72.4**	66.2	52.1	62.9	**74.2**	42.4	54.3	58.6
18:1ω8*c*	**-**	-	-	-	**-**	1.1	-	-
18:1ω9*c*	**-**	1.1	-	1.1	**-**	-	-	-
11-Methyl 18:1ω7*c*	**4.2**	16.7	9.6	19.1	**tr**	21.7	6.7	tr
19:0 cyclo ω8*c*	**10.3**	-	20.7	4.3	**13.3**	9.7	16.5	13.5
20:0	**-**	-	-	-	**-**	-	-	1.2
Unkown (ECL 17.610)	**-**	tr	tr	tr	**-**	-	-	-
Unkown (ECL 18.796)	**tr**	-	-	-	**tr**	tr	-	-
Unkown (ECL 18.846)	**-**	tr	tr	1.4	**-**	-	-	-
Summed feature 2[a]	**tr**	1.1	tr	tr	**-**	-	-	-
Summed feature 3[a]	**tr**	2.7	3.2	1.4	**-**	1.7	-	tr
Summed feature 4[a]	**-**	-	-	-	**3.0**	-	-	-

[a] Summed feature 2 comprised 14:0 3-OH and/or 16:1 iso I, summed feature 3, 16:1 ω7*c* and/or 15:0 iso 2-OH, and summed feature 4, 17:1 anteiso B and/or 17:1 iso I.

colonies (Gray and Rolfe, 1990). The novel strain grown on YMA, however, did not produce slimy colonies that were shown in the strains: *Mesorhizobium huakuii* IAM 14158^T and *Mesorhizobium plurifarium* LMG 11892^T as a positive control. A *nifH* gene encoding denitrogenase reductase, the key component of the nitrogenase enzyme complex, was also searched by PCR amplification using a *nifH*-specific primer set: PolF and PolR, designed by Poly *et al*. (2001). A *nifH* gene also was not detected from the extracted DNA of the novel strain; this suggests that novel strain has no ability to fix nitrogen that is the functional traits of rhizobia. Taken together with polyphasic phylogenetic analysis with the 16S rRNA gene sequence, genomic DNA G+C content, major isoprenoid quinone, fatty acid profile, and results from physiological and biochemical tests, it is proposed that strain N8^T should be classified as a novel species of the new genus *Aliihoeflea* in the family *Phyllobacteriaceae*, for which the name *Aliihoeflea aestuarii* gen. nov., sp. nov. is proposed.

Description of *Aliihoeflea* gen. nov.

Aliihoeflea (A.li.i.ho.e.fle'a. L. adj. and pronoun alius, other, another, different; N.L. fem. n. *Hoeflea*, a bacterial genus name; N.L. fem. n. *Aliihoeflea*, the other *Hoeflea*).

Cells are Gram-negative and rod-shaped. Catalase- and oxidase-positive. Predominant fatty acids are $C_{18:1}$ ω7*c* and $C_{19:0}$ cyclo ω8*c*. Major isoprenoid quinone is ubiquinone-10. The genomic DNA G+C content of the type species is about 53 mol%. The type species is *Aliihoeflea aestuarii*.

Description of *Aliihoeflea aestuarii* sp. nov.

Aliihoeflea aestuarii (a.es.tu.a.ri'i. L. gen. n. *aestuarii*, of a tidal flat).

Cells are rod-shaped (0.50~0.75 μm wide and 1.25~1.50 μm long). Colonies on MA plates are circular with entire margin, convex, shiny, cream-colored, and around 0.5~1.0 mm in diameter after 2 days incubation at 30°C. Growth also occurs on complex media like TSA, LA, and YMA, but not on R2A. Growth occurs at 17~37°C, but not 15°C and 40°C, with optimal growth occurring at 30°C. The strain does not require NaCl and growth occurs up to 8% (w/v) NaCl with optimal growth occurring at 1% NaCl, but growth does not occur at 10% NaCl. The strain is catalase- and oxidase-positive, and does not reduce nitrates to nitrites or nitrogen. Indole production does not occur. Glucose fermentation and hydrolysis of starch, aesculin, gelatin, and PNPG (*p*-nitrophenyl-β-D-galactopyranoside) do not occur. Urease-positive and arginine dihydrolase-negative. Sole carbon sources utilized for growth include glycogen, Tween 80, L-arabinose, D-fructose, pyruvic acid methyl ester, succinic acid monomethyl-ester, acetic acid, α-hydroxybutyric acid, β-hydroxy-

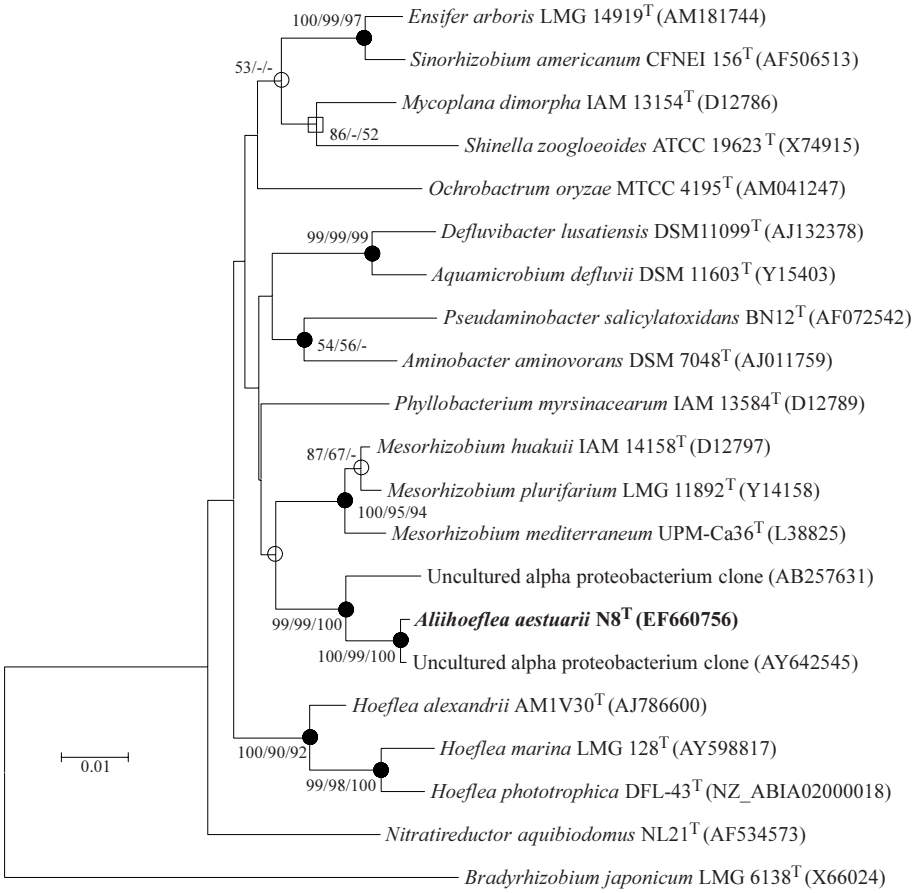

Fig. 1. Neighbor-joining tree showing the phylogenetic positions of *Aliihoeflea aestuarii* N8^T and related species based on 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. Filled circles, open circles and a square indicate generic branches that were also recovered using the maximum-parsimony and maximum-likelihood algorithm, using the maximum-parsimony algorithm, and maximum-likelihood algorithm, respectively. Numbers at nodes indicate bootstrap values as calculated by neighbor-joining/ maximum-parsimony/ maximum-likelihood probabilities in percent. Bootstrap values greater than 50% are shown at the branch points. Bar, 0.01 accumulated changes per nucleotide.

butyric acid, γ-hydroxybutyric acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, succinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, L-serine, inosine, uridine, and thymidine. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphtol-AS-BI-phosphohydrolase; and negative for lipase (C14), acid phospatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Predominant fatty acids are $C_{18:1}$ *ω7c* and $C_{19:0}$ cyclo *ω8c* (Table 2). The major isoprenoid quinone is ubiquinone-10 and genomic DNA G+C content is 53.4 mol%. The type strain is N8^T (= KCTC 22052^T= JCM 15118^T= DSM 19536^T) which was isolated from tidal flat sediment in Yeosu (34°47'26" N 127°34'01" E), Republic of Korea.

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