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Aureivirga callyspongiae sp. nov., Isolated from Marine Sponge *Callyspongia elegans*

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A Gram-negative, aerobic, motile by gliding, and rod-shaped marine bacterium, designated CE67^T was isolated from the marine sponge *Callyspongia elegans* on Biyang-do in Jeju Island. The CE67^T strain grew optimally at 25℃, pH 7.5, and in the presence of 2–3% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequence showed that strain CE67^T was related to the genus *Aureivirga* and had the highest 16S rRNA gene sequence similarity to the *Aureivirga marina* VIII.04^T type strain (96.3%). The primary fatty acids (>10%) of strain CE67^T were iso-$C_{15:0}$ (35.3%) and iso-$C_{17:0}$ 3OH (21.8%). The polar lipid profile of strain CE67^T contained phosphatidylethanolamine, unidentified aminolipids, and unidentified lipids. The predominant menaquinone was MK-6. The DNA G+C content was 29.1 mol%. Based on the polyphasic taxonomic analysis, strain CE67^T was determined to be a representative novel species of the genus *Aureivirga* for which we propose the name *Aureivirga callyspongiae* sp. nov., whose strain type is CE67^T (=KCTC 42847^T =JCM 34566^T).

Keywords: ***Aureivirga*, marine sponge, novel species, taxonomy**

Introduction

Sponges are known to harbor various microorganisms at a high density, which comprise 40 to 60% of their biomass [1, 2]. We isolated cultivable marine bacteria by screening for bacterial diversity in the sponge *Callyspongia elegans*, and identified novel bacterial taxa. One of the isolates, strain CE67^T, was selected for a later taxonomic experiment. Comparative 16s rRNA gene sequence analysis indicated that the novel CE67^T strain was phylogenetically most closely related to members of the genera *Aureivirga* [3], *Namhaeicola* [4], *Actibacter* [5], and *Lutimonas* [6, 7]. The genus *Aureivirga* in the phylum *Bacteroidetes* is a new member of the family *Flavobacteriaceae*, and was first reported by Haber *et al*. in 2013 [3]. It was isolated from the marine sponge *Axinella verrucosa* collected from the coast of Israel. At the time of this writing, one species belongs to the genus *Aureivirga*, known as *Aureivirga marina*. *A. marina* is Gram-negative, non-motile, aerobic, and forms golden-brown colonies. The genus also grows at 15–37℃, pH 6.0–9.0, and 2–5% (w/v) NaCl. The major fatty acid (>50%) is iso-$C_{15:0}$, and the major respiratory quinone is MK-6. In addition, the DNA G+G content is 30.7 mol%. In this study, we investigated the bacteria CE67^T isolated from the sponge *Callyspongia elegans* collected on Biyang-do in Jeju Island using polymorphic experiments including morphological, physiological, chemotaxonomic, and 16S rRNA gene sequence analysis.

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Materials and Methods

Isolation

Sponge samples were collected by scuba diving at a depth of 10 m in Biyang-do, Jeju. To isolate marine bacteria, homogenized sponge tissue samples were serial

diluted (~10^{-7}) with a sterile 0.85% (w/v) NaCl solution. Each diluted solution was plated on marine agar (MA) medium and incubated for 7 days at 25℃. After separating a single colony, it was suspended in marine broth containing 20% (v/v) glycerol and stored at -80℃ for use in further experiments. The most closely related species, *A. marina* LMG26721^T, was obtained from the Belgian Orchestrated Microbial Collection (BCCM), and was used as the type strain.

16S rRNA gene sequencing and phylogenetic analyses

For 16S rRNA and genome sequencing, genomic DNA of strain CE67^T was extracted as described by Wilson [8]. The 16S rRNA gene was amplified by PCR using the universal primers 27F and 1522R [9]. The PCR product was cloned into pGEM®-T Easy Vector Systems (Promega) and sequenced by Genotech (Korea). The full sequence of the 16S rRNA gene (1,510 bp) was compiled with SeqMan software (DNASTAR). The 16S rRNA sequences for related taxa were obtained from the EzTaxon-e server (www.Ezbiocloud.net) [10] and aligned using CLUSTAL_X [11]. Gaps were edited using the BioEdit program [12]. The phylogenetic tree was reconstructed with three algorithms: neighbor-joining (NJ)[13], maximum-likelihood (ML) [14], and maximum-parsimony (MP) [15, 16] using MEGA 7.0 software [17], and bootstrap values were calculated based on 1000 replicates [18]. The Jukes-Cantor model [19] was used to calculate evolutionary distances.

Genomic analysis

Whole genome sequencing of strain CE67^T and type strains were carried out by the Macrogen (Republic of Korea) sequencing service. The sequencing library was prepared using the TruSeq DNA PCR-Free kit according to the manufacturer's instructions. The genome sequence data were obtained using the Illumina platform and de novo assembly was performed with SPAdes version 3.13.0. The DNA G+C content of strain CE67^T was determined from whole genome sequences. Moreover, the average nucleotide identity (ANI) value was calculated using the ANI calculator provided by the Ezbiocloud server [20]. Whole genome sequences were submitted to the GenBank database.

Morphology and physiological and biochemical characterization

The morphology of the cells was observed by transmission electron microscopy (SUPRA66VP, ZEISS). Motility was assessed using marine broth supplemented with 0.5% agar, according to the method of Bowman [21]. Gram staining was performed using a Gram staining kit (BD Science, USA) according to the manufacturer's instructions. Catalase activity was detected by observing oxygen bubble production using H_2O_2 (3%, v/v), and oxidase activity was determined using an oxidase reagent (bioMérieux, UK). Anaerobic growth was observed under anaerobic conditions using ANAEROPACK (Oxoid, UK) in an anaerobic JAR (Thermo Fisher Scientific, USA) with MA medium at 25℃ for 2 weeks.

Growth was observed at various temperatures in MA (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50℃), and pH sensitivity measurements were carried out in marine broth adjusted to pH 4.0–11.0 (at intervals of 0.5 pH units). The pH was adjusted using citric acid/sodium citrate buffer, KH_2PO_4/K_2HPO_4, and $NaHCO_3/Na_2CO_3$ as buffering systems. NaCl tolerance was tested in synthetic marine ZoBell medium (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, and 15 g agar in 1 L distilled water) [22] supplemented with 0–10% (w/v) NaCl (at 1% intervals). The hydrolysis tests of strain CE67^T were performed using MA containing starch (1%, w/v), casein (3%, w/v), Tween 20, 40, 60 and 80 (1%, w/v), cellulose (0.5%, w/v), and DNase (1%, w/v) [23]. The enzyme activities and biochemical characteristics of CE67^T and *A. marina* LMG26721^T were determined using API ZYM and API 20 NE kits (bioMérieux) according to the manufacturer's instructions. These tests were carried out at 25℃ for 5 days. Strain CE67^T and *A. marina* LMG26721^T were used to perform experiments in this study. Other types of strains, *Namhaeicola litoreus* DPG-25^T, *Actibacter sediminis* JC2129^T, *Lutimonas vermicola* IMCC1616^T, and *Lutimonas saemankumensis* SMK-142^T, are described in Table 1 with reference to data from the references [4–7].

Chemotaxonomy

For the analysis of polar lipids and quinone, strain CE67^T was incubated in MA for 5 days at 25℃, and freeze-dried cells were used in the experiment. Polar lip-

ids of strain CE67^T were extracted as described by Minnikin *et al*. [24] and detected using two-dimensional thin layer chromatography (TLC). Extracted polar lipids were separated using chloroform/methanol/water (65:25:4, by volume) and chloroform/ methanol/acetic acid/water (80:12:15:4, by volume) for mobile phases in the first and second dimension, respectively. Plates were stained with four detection reagents, molybdophosphoric acid (for total lipids), ninhydrin (for lipids containing free amino groups), molybdenum blue (for lipids containing phosphorus), and α-naphthol (for glycolipids) [25]. Then, the polar lipid patterns were analyzed and compared. Analysis of the respiratory quinone was performed by reversed phase high-performance liquid chromatography [26].

For a comparison of cellular fatty acid composition, strain CE67^T and one type strain were harvested from MA after cultivation at 25℃ for 5 days. According to the protocol for the Sherlock Microbial Identification System (MIDI; version 6.1), cellular fatty acids were saponified, methylated, and extracted. Methylated fatty acids were analyzed by gas chromatography and identified using the TSBA6 database [27].

Table 1. Differential phenotypic characteristics of Strain CE67^T and the type strains of *A. marina* and related genera of family *Flavobacteriaceae*. Strain: 1, CE67^T (data from this study); 2, *Aureivirga marina* LMG 26721 ^{T} (data from this study); 3, *Namhaeicola litoreus* DPG-25^T (data from Jung *et al*., 2012); 4, *Actibacter sediminis* JC2129^T (Kim *et al*., 2008); 5, *Ltimonas saemankumensis* SMK-142^T (Yoon *et al*., 2008); 6, *Lutimonas vermicola* IMCC1616^T (Yang *et al*., 2007). +, Positive; -, negative; w, weak; ND, not determined. All strains are positive for activities of alkaline phosphatase and acid phosphatase. All strains are negative for activities of lipase (C14), β-glucuronidase, α-fucosidase and nitrate reduction.

Characteristic	1	2	3	4	5	6
Oxidase	+	+	+	+	+	-
Catalase	+	+	+	+	+	-
Growth range at/in :						
10℃	-	-	-	+	+	+
37℃	w	+	+	+	-	+
pH 5.0	-	+	-	+	-	+
1% NaCl	-	+	+	+	+	+
4% NaCl	-	w	+	+	+	+
Utilization of citrate	-	+	-	-	-	+
Hybrolysis :						
Gelatin	+	+	-	+	-	+
STA (Starch)	+	-	+	+	+	+
CAS (casein)	-	+	-	-	-	
Aesculin	-	-	+	+	-	ND
Tween 80	+	+	-	+	-	-
Enzyme activity (API ZYM):						
Esterase (C4)	+	-	-	+	-	+
Cystine arylamidase	+	+	-	-	+	+
Trypsin	+	+	-	-	-	-
α-chymotrypsin	+	+	-	+	-	-
Naphtol-AS-BI-phosphohydrolase	+	+	-	+	-	-
β-galactosidase	-	+	-	-	-	-
α-glucosidase	-	-	-	+	-	-
N-acetyl-β-glucosaminidase	-	-	+	-	+	+
DNA G+C content (mol%)	29.3	30.7	39.9	43-45	37.2	40.1

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Results and Discussion

Strain CE67^T was aerobic, Gram-negative, and formed light brown rods (0.3–0.5 μm × 1.6–3.1 μm). The colonies were observed to be opaque and circular after growth for 5 days at 25℃ (optimum) on an MA plate. Morphological, physiological, and biochemical characteristics were compared with those of the reference strain *A. marina* VIII.04^T, as listed in Table 1.

The length of the 16S rRNA gene sequence of strain

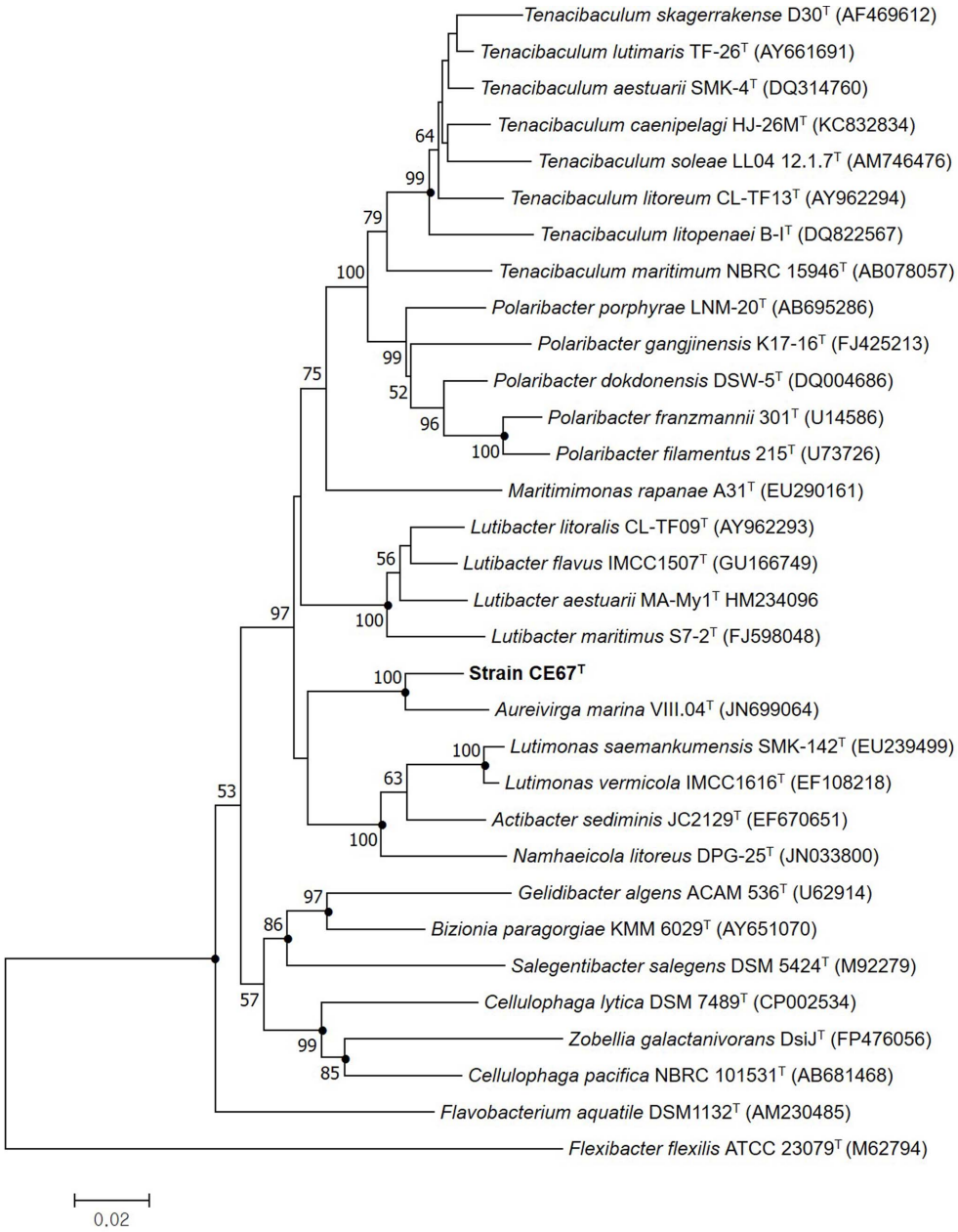

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain CE67^T and closely related taxa. Bootstrap values (based on 1000 resamplings) > 50% are shown at branching points. Filled circles indicate that corresponding nodes were branches found in phylogenetic consensus trees generated with the Maximum-Likelihood and Maximum-Parsimony method. *Flexibacter flesilis* ATCC 23079^T (M62794) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of strain CE67^T and the type strain of *Aureivirga marina* LMG 26721^T.
Strain: 1, CE67^T (data from this study); 2, *Aureivirga marina* LMG 26721^T. All data are from this study. The composition amount of at least 0.5% of the total cellular fatty acids is shown. Summed features 3 contained iso- $C_{15:0}$ 2-OH/$C_{16:1}$ *ω7c*.ECL, Equivalent chain length. -, not detected; tr, Traces (<0.5%).

Fatty acid	1	2
Staight-chain		
$C_{13:0}$	tr	tr
$C_{14:0}$	0.8	0.5
$C_{16:0}$	2.33	2.88
Branched		
iso-$C_{13:0}$	0.5	0.5
iso-$C_{14:0}$	tr	-
iso-$C_{15:0}$	35.3	35.2
anteiso-$C_{15:0}$	0.5	tr
iso-$C_{15:1}$ G	3.9	7.0
iso-$C_{16:0}$	0.7	1.6
iso-$C_{17:0}$	tr	0.9
Hydroxylated		
$C_{15:0}$ 3OH	2.8	2.2
$C_{16:0}$ 3OH	3.5	1.7
$C_{17:0}$ 3OH	0.9	0.7
iso-$C_{15:0}$ 3OH	9.7	7.9
iso-$C_{16:0}$ 3OH	1.2	0.7
iso-$C_{17:0}$ 3OH	21.8	18.1
Unsaturated		
$C_{15:1}$ *ω6c*	1.3	0.6
$C_{17:1}$ *ω6c*	2.0	1.5
$C_{17:1}$ *ω8c*	tr	0.6
iso-$C_{17:1}$ *ω9c*	1.4	4.5
$C_{18:1}$ *ω5c*	0.7	tr
Unknown fatty acid		
ECL 13.565	2.0	5.0
ECL 16.582	0.9	0.8
Summed features		
3	4.9	5.1

CE67^T was 1,510 bp, and phylogenetic analysis revealed the highest similarity to *A. marina* VIII.04^T (96.3%). Also, strain CE67^T closely clustered with members of the genera *Namhaeicola*, *Actibacter* and *Lutimonas*. The Family *Flavobacteriaceae,* and formed a distinct lineage with to *A. marina* VIII.04^T (Fig. 1). The whole genome of strain CE67^T was 4,818,964 bp, containing 48 contigs

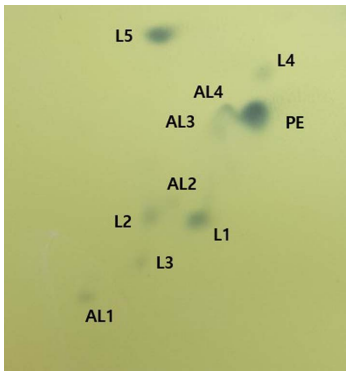

Fig. 2. Polar lipid profiles of strain CE67^T. Total polar lipids were spray with molybdophosphoric acid reagent. PE, phosphatidylethanolamine; AL, unknown aminolipids; L1-2, unknown lipids.

with an N50 length of 196,835 bp. The sequencing depth of the ANI value between strain CE67^T and type strain *A. marina* VIII.04^T was 82.8%, which was lower than the standard cut-off of 95–96% for species identity [28]. The major fatty acids (>3% of the total fatty acids) of CE67^T were $C_{15:0}$ (35.3%) and $C_{17:0}$ 3OH (21.8%), iso-$C_{15:0}$ 3OH (9.7%), summed feature 3 (4.9%), iso-$C_{15:0}$ G (3.9%), and $C_{16:0}$ 3OH (3.5%) (Table 2). The cellular fatty acids of strain CE67^T and the most closely related species are shown in Table 2. The predominant fatty acids of strain CE67^T and *A. marina* VIII.04^T were $C_{15:0}$ and $C_{17:0}$ 3OH. When the fatty acid composition was compared, the pattern was similar to that of the reference type strain, but the proportion of some fatty acids differed. The major isoprenoid quinone was MK-6. The polar lipids of strain CE67^T consisted of phosphatidylethanolamine (PE), four unknown aminolipids, and five unknown lipids (Fig. 2). The CE67^T polar lipid profiles were the most similar to the pattern observed in the type strain *A. marina*, as PE was detected in both. However, some aminolipids and lipids were found only in strain CE67^T. The value of the DNA G+C content was calculated as 29.3 mol%, which is lower than that reported for the genus *Aureivirga* (31.1 mol%). Based on the results of polyphasic taxonomic data, strain CE67^T represents a new species of the genus *Aureivirga*, for which the name *Aureivirga callyspongiae* sp. nov. is proposed.

Description of *Aureivirga callyspongiae* sp. nov.

Aureivirga callyspongiae (cal.ly.spon'gi.ae. N.L. gen. n.

callyspongiae of the sponge *Callyspongia*) strain CE67^T is rod-shaped, Gram-negative, aerobic, and exhibiting gliding motility. The organisms were light-brown, approximately 0.3–0.5 μm wide and 1.6–3.1 μm long and opaque after 5 days of incubation at 25℃ on marine agar. Growth occurs at 15–35℃ (optimum, 25℃), pH 5.5–9.5 (optimum, pH 7.5), and in 2–3% of NaCl (w/v) (optimum, 2%). Catalase and oxidase tests were positive. The strain also tested positive for hydrolysis of starch, Tween 40, 60, 80, and DNA, but negative for that of casein, cellulose, aesculin, Tween 20, and urea. In the 20 NE tests, a positive result was found for the hydrolysis of gelatin, but nitrate reduction, indole production, glucose fermentation, the presence of arginine dihydrolase, and the assimilation of D-glucose, D-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, gluconate, caprate, adipate, malate, citrate, and phenylacetate were negative. In the ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase were present, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were absent. The main fatty acids of strain CE67^T are iso-$C_{15:0}$ and iso-$C_{17:0}$ 3OH. The polar lipid profile showed the presence of PE, four unidentified aminolipids, and five unidentified lipids. The major respiratory quinone is menaquinone-6 (MK-6). The DNA G+C content is 29.1 mol %. The type strain CE67^T (=KCTC 42847^T =JCM 34566^T) was isolated from marine sponge *Callyspongia elegans* in Jeju Island, Republic of Korea.

GenBank accession number

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and whole genome sequence of *Aureivirga callyspongiae* CE67^T are KT596060 and JADOTV000000000.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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