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Amylibacter kogurei sp. nov., a novel marine alphaproteobacterium isolated from the coastal sea surface microlayer of a marine inlet

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Abstract

A novel Gram-negative bacterium, designated 4G11^T, was isolated from the sea surface microlayer of a marine inlet. On the basis of 16S rRNA gene sequence analysis, the strain showed the closest similarity to *Amylibacter ulvae* KCTC 32465^T (99.0 %). However, DNA–DNA hybridization values showed low DNA relatedness between strain 4G11^T and its close phylogenetic neighbours, *Amylibacter marinus* NBRC 110140^T (8.0±0.4 %) and *Amylibacter ulvae* KCTC 32465^T (52.9±0.9 %). Strain 4G11^T had $C_{18:1}$, $C_{16:0}$ and $C_{18:2}$ as the major fatty acids. The only isoprenoid quinone detected for strain 4G11^T was ubiquinone-10. The major polar lipids were phosphatidylglycerol, phosphatidylcholine, one unidentified polar lipid, one unidentified phospholipid and one unidentified aminolipid. The DNA G+C content of strain 4G11^T was 50.0 mol%. Based on phenotypic and chemotaxonomic characteristics and analysis of the 16S rRNA gene sequence, the novel strain should be assigned to a novel species, for which the name *Amylibacter kogurei* sp. nov. is proposed. The type strain of *Amylibacter kogurei* is 4G11^T (KY463497=KCTC 52506^T=NBRC 112428^T).

The genus *Amylibacter* from the family Rhodobacteracaea was first proposed by Teramoto and Nishijima [1] as an aerobic rod-shaped bacterium that grows well in the presence of starch. At the time of writing, the genus *Amylibacter* is represented by three validated species isolated mainly from marine environments. *Amylibacter marinus* was first isolated from the surface seawater [1]. *Amylibacter ulvae* was thereafter isolated from green algae [2] followed by *Amylibacter cionae* isolated from sea squirt [3].

The sea surface microlayer (SML) lies between the air–sea boundary and is widely defined as the top 1 mm of the ocean surface [4]. The physicochemistry of the SML is distinct from the underlying water and is usually enriched with different organic compounds [4]. Previous studies have also shown that bacteria in the SML are different from those in the underlying water directly below it [5, 6]. This paper describes a new member within the genus *Amylibacter*, designated as strain 4G11^T, isolated from the SML of a coastal inlet.

Strain 4G11^T was isolated from an SML water sample collected using a drum sampler positioned [7] near the pier of the University of Tokyo's Misaki Marine Biological Station (35° 09.5′ N 139° 36.5′ E) at Misaki, Japan. Aliquots of 100 µl seawater from the SML were plated onto 1/10–strength ZoBell agar medium (0.5 g peptone, 0.1 g yeast extract, 15 g agar in 800 ml natural seawater aged for at least 1 year and 200 ml distilled water). Initial incubation was carried out at 20 °C for 4 days. Routine cultivation for subsequent characterizations was performed at 25 °C on 1/2–strength marine agar 2216 (Difco) supplemented with 1.0 % NaCl (w/v), hereby known as 1/2 MA. The isolate was preserved at −80 °C after suspension of the culture in 1/2-strength marine broth supplemented with 1 % NaCl (w/v) and 20 % (w/v) glycerol. *A. marinus* NBRC 110140^T and *A. ulvae* KCTC 32465^T obtained from the NITE Biological Resource Centre (NBRC) and the Korean Collection for Type Cultures (KCTC), respectively, were used as reference strains and were characterized under the same conditions as strain 4G11^T for comparative analyses.

The nearly full-length 16S rRNA gene of strain 4G11^T was amplified using the 27F/1492R universal primer set [8] and

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Keywords: *Amylibacter*; MK10; sea surface microlayer.
Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; SML, sea surface microlayer.
The whole-genome shotgun project for 4G11^T has been deposited in DDBJ/EMBL/GenBank under the accession number MDGM01000000. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence for strain 4G11^T is KY463497.
Two supplementary figures are available with the online version of this article.

sequenced using an ABI 3130xl DNA sequencer. The sequence obtained (1282 bp) was subjected to BLAST search [9] in the GenBank database [10] and the EzBioCloud 16S rRNA gene database [11]. Sequences of related type strains were collected for inclusion in the phylogenetic analysis. Alignments of multiple sequences were performed using the CLUSTAL_W option within MEGA 7.0 software [12]. Phylogenetic and molecular evolutionary analyses were also conducted with MEGA 7.0 using evolutionary distances calculated from Kimura's two-parameter model [13] and clustered using the neighbour-joining (NJ) [14], maximum-likelihood (ML) [15] and maximum-parsimony (MP) [16] methods. The robustness of the phylogenetic tree was assessed by means of bootstrap resampling based on a 1 000 replicates [17]. The 16S rRNA gene sequence of strain 4G11^T showed highest similarity to *A. ulvae* KCTC 32465^T (99.0 %). Phylogenetic trees obtained by the NJ, MP and ML methods revealed the highest affiliation between 4G11^T and *A. ulvae* KCTC 32465^T at a bootstrap confidence levels of 85 % (NJ), 91 % (ML) and 94 % (MP) (Fig. 1). The DNA–DNA hybridization (DDH) test was carried out in four replicates according to the method described by Ezaki *et al.* [18] to measure the degree of genetic similarity between strain 4G11^T and the two reference strains, *A. marinus* NBRC 110140^T and *A. ulvae* KCTC 32465^T. DNA–DNA relatedness data showed that strain 4G11^T is distinct from *A. ulvae* KCTC 32465^T (52.9±0.9 % similarity; reciprocal DDH 49.7±1.9 %) and *A. marinus* NBRC 110140^T (8.0 ±0.4 % ; 6.4±0.4 %).

Cell morphology of strain 4G11^T was examined according to Børsheim *et al.* [19] using mid-log phase cells cultured in 1/2-strength marine broth supplemented with 1 % NaCl. After staining the carbon and parlodion-coated copper grid (150 mesh, Stork Veco International) for 30 s with 2 % uranyl acetate, at least 50 fields were examined at ×75 000 magnification using a JEM-1400EX transmission electron microscope (JEOL). Cells of strain 4G11^T were rod-shaped ranging between 0.76–2.47 µm in length and 0.39–0.70 µm in diameter (n=50, Fig. S1, available in the online version of this article). Swarming and gliding/twitching motilities were examined using 0.1–0.5 % 1/2 soft MA (at 0.1 % increments) and 1.0 % 1/2 MA as described by Harshey [20]. Strain 4G11^T neither showed swarming nor twitching/gliding motilities. Gram staining performed on cells grown on 1/2 MA for 2 days using the Gram Stain Kit (BD) showed that the strain was Gram-negative. Growth under anaerobic condition was determined after incubation with AnaeroPack (Mitsubishi Gas Chemical Co.) on 1/2 MA for 4 weeks at 25 °C. Strain 4G11^T showed no growth under these anaerobic conditions. Growth at different temperatures (5–40 °C at 5 °C intervals) were assessed for 1 week on modified 1/2 MA. The modified 1/2 MA contained (per litre distilled water) 2.5 g peptone (BD), 0.5 g yeast extract (Bacto yeast

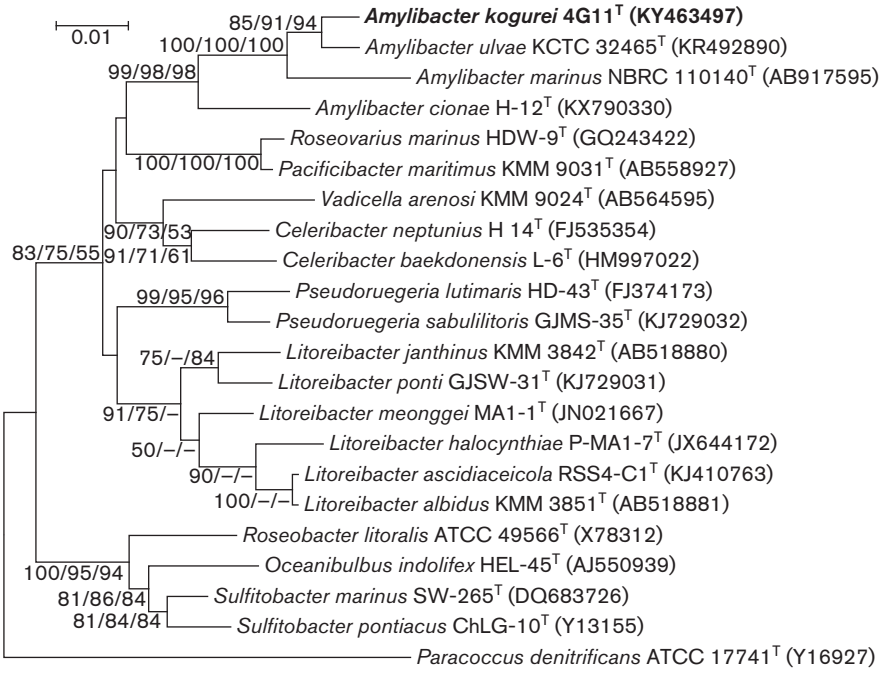

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain 4G11^T. GenBank accession numbers of the type strains used in the phylogenetic analysis are given in parentheses. Bootstrap values based on 1000 replicates are indicated at branch points. '–' indicates branch with bootstrap values less than 50 %. Bootstrap values are listed in the order of neighbour-joining, maximum-likelihood and maximum parsimony methods. The tree was rooted using *Paracoccus denitrificans* ATCC 17741^T as the outgroup.

extract, BD), 0.05 g ferric citrate, 0.9 g $CaCl_2.2H_2O$ and 3.0 g $MgCl_2.6H_2O$ and 1.0 % NaCl (w/v). The medium was adjusted to pH 7.5. Salt tolerance under different concentrations of NaCl ranging from 0 to 10 % w/v (at 1 % intervals) and pH tolerance ranging from pH 4–13 (at 1 pH unit intervals) were also tested using modified 1/2 MA that was incubated at 25 °C. Strain 4G11^T grew well with 1–7 % NaCl (optimally at 1–5 %), pH 5–12 (pH 7–10) and within the temperature range of 5–30 °C (15–30 °C).

Tests for catalase and oxidase activities were determined on cells incubated on 1/2 MA for 2 days. Catalase activity was tested by observing if bubbles were produced after the addition of 3 % hydrogen peroxide solution (Sigma) to colonies. Oxidase activity was tested using cytochrome oxidase paper (Nissui Pharmaceutical Co.). The strain was oxidase- and catalase-positive.

The degradation of DNA was tested using DNase agar (Oxoid) and flooding the plates with 1 M HCl after incubation at 25 °C for three days. Degradation of soluble starch and CM-cellulose were tested according to Atlas [21] and Bowman [22], respectively, after incubation at 25 °C for 3 days. Chitin degradation was determined according to Sakazaki *et al.* [23] at TechnoSuruga Laboratory Co., Ltd. Strain 4G11^T was able to hydrolyse DNA and starch but not CM-cellulose and chitin. Additional biochemical properties of strain 4G11^T and the reference strains, *A. marinus* NBRC 110140^T and *A. ulvae* KCTC 32465^T were determined using API ZYM, API 20E, API 20NE and API 50CH (bioMérieux). Cells grown on 1/2 MA for 3 days were suspended in respective API suspension media supplemented with 3 % (w/v) NaCl (final concentration). The API strips were incubated at 25 °C and read after 48 h for API 20E, API 20NE and API 50CH strips and 24 h for API ZYM strips. The API results are listed in Table 1 and in the species description.

Fatty acids, isoprenoid quinones, polar lipids and G+C content analyses were also performed for strain 4G11^T, *A. marinus* NBRC 110140^T and *A. ulvae* KCTC 32465^T. Cells for fatty acid analysis were grown for 3 days at 25 °C on 1/2 MA. Fatty acid methyl esters were prepared according to the standard protocol of the Sherlock Microbial Identification System Version 6.0 (MIDI) with the TSBA6 database. The cellular fatty acid composition for strain 4G11^T, *A. marinus* NBRC 110140^T and *A. ulvae* KCTC 32465^T are listed in Table 2. The major cellular fatty acids of strain 4G11^T were $C_{18:1}$ (81.9 % of total), $C_{16:0}$ (8.7 %) and $C_{18:2}$ (6.7 %). The fatty acid profile for strain 4G11^T and the two other *Amylibacter* reference strains were similar in the prevalence of the major fatty acid $C_{18:1}$, but can be differentiated from the reference strains by higher proportions of $C_{16:0}$ and $C_{18:2}$ as well as the absence and presence of $C_{14:0}$ and $C_{18:0}$ fatty acids respectively. Isoprenoid quinones, polar lipids and G+C content analyses for strain 4G11^T were performed at TechnoSuruga Co., Ltd. Cells for these analyses were grown for 3 days at 25 °C on 1/2 MA. Isoprenoid quinones were extracted using methanol/isopropanol (3 : 1, v/v) according to Blight and Dyer [24] and analysed by the ACQUITY UPLC system (Waters). Ubiquinone-10 (Q-10), which is the predominant ubiquinone of the genus *Amylibacter* as well as the majority of the members of the class *Alphaproteobacteria*, was the predominant ubiquinone detected in strain 4G11^T (Table 1). Polar lipids were extracted and separated using two-dimensional thin-layer chromatography and identified according to previously published procedures [25]. The spots for total polar lipids were identified by spraying the ultra-performance thin-layer chromatography plates with 5 % (w/v) molybdophosphoric acid in ethanol and incubating at 180 °C for 20 min. Other polar lipids present were detected using specific spray reagent (α-naphthol for glycolipids, ninhydrin for aminolipids, molybdenum blue for phospholipids and Dragendorff reagent for phosphatidylcholine). The major polar lipids of strain 4G11^T were phosphatidylglycerol, phosphatidylcholine, one unidentified polar lipid, one unidentified phospholipid and one unidentified aminolipid; three unidentified polar lipids (UL2–4), were detected as minor polar lipids (Fig. S2). For the measurement of genomic DNA G+C content, genomic DNA from cells cultured on 1/2 MA for 3 days at 25 °C was extracted and purified according to Kawamura [26]. Genomic DNA was degraded enzymatically into nucleosides using the DNA-GC kit (Yamasa Corporation) according to Suzuki [27] and genomic DNA G+C content of the deoxyribonucleosides was determined by reverse-phase high-performance liquid chromatography [28]. The DNA G+C content of strain 4G11^T was 50.0 mol%, which is within the reported range (50.0–52.7 mol%) for other species of the genus *Amylibacter*.

DNA extraction and genome sequencing for strain 4G11^T were performed according to Wong *et al.* [29] on a MiSeq (Illumina) sequencing platform using a library prepared by KAPA HyperPlus kit (Kapa Biosystems). The resulting MiSeq paired end reads were assembled using Platanus version 1.2.4 [30] and the resulting sequences were subsequently annotated automatically using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) [31] and reviewed with Rapid Annotation using Subsystem Technology (RAST) version 2.0 (http://rast.nmpdr.org). The strain 4G11^T genome sequence consisted of 16 scaffolds (2 983 050 bp in total, N_{50}=298 011 bp) with a median read coverage of 183.0×. The PGAAP identified 3169 genes including 3065 protein-coding sequences (CDSs), 36 tRNA, four non-coding RNA genes and 28 pseudogenes. The RAST annotation also identified 2962 CDSs and 431 sets of functional roles or susbsystems in the RAST database.

The results of the phylogenetic analysis indicated that strain 4G11^T represents a member within the genus *Amylibacter* but the low DDH values between strain 4G11^T and the other closest relatives within the genus *Amylibacter* suggested that strain 4G11^T represents a novel species. Although strain 4G11^T shared many common traits with its closest relatives, *A. marinus* and *A. ulvae*, there were also differences in the phenotypic and chemotaxonomic observations between strain 4G11^T and the two reference type strains, such as the ability to grow at

Table 1. Differential characteristics between strain 4G11^T and its closest phylogenetic relatives, *Amylibacter marinus* NBRC 110140^T and *Amylibacter ulvae* KCTC 32465^T

Strains: 1, 4G11^T; 2, *Amylibacter marinus* NBRC 110140^T and 3, *Amylibacter ulvae* KCTC 32465^T. +, Positive; –, negative; w, weakly positive. PG, phosphatidylglycerol; PC, phosphatidylcholine; UL, unidentified polar lipid; PL, unidentified phospholipid; AL, unidentified aminolipid. All strains are positive for catalase and oxidase activities; DNA degradation; urease and aesculin hydrolysis; enzyme activities of alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase and cysteine arylamidase activities (API ZYM); and acid production from mannose, L-rhamnose, arbutin, salicin, cellobiose, melezitose, raffinose and 5-keto-gluconate (API 50 CH). All strains are negative for reduction of nitrate to nitrite; fermentation and assimilation of glucose; nitrite reduction; gelatin hydrolysis; cellulose utilization; indole production; trypsin, α-chymotrypsin, acid phosphate, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities (API ZYM); L-tryptophane, arginine dihydrolase, L-arabinose, mannose, mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid assimilation (API 20NE) and glycerol, adonite, D-galactose, dulcitol, sorbitol, α-methyl D-mannoside, melibiose, turanose, D-lyxose, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate (API 50CH).

Characteristic	1	2	3
Temperature range for growth (°C)	5–30	5–25	10–40
Salinity range (% NaCl, w/v)	1–7	1–3	1–10
pH range for growth (pH)	5–12	4–11	4–11
Starch hydrolysis	+	+	–
ONPG hydrolysis	+	–	+
Acetoin production	w	–	w
Enzyme activities (API ZYM):			
Naphthol-AS-BI-phosphohydrolase	+	w	–
α-Glucosidase	w	–	–
Assimilation of (API 20NE):			
4-Nitrophenyl-β-D-galactopyranoside	+	–	+
Acid production from (API 50CH):			
Sucrose	w	–	–
L-Arabinose, ribose, inositol	w	–	+
D-Glucose, D-fructose, lactose, trehalose, inulin	w	–	w
Sorbose	–	w	w
Methyl α-D-glucoside, *N*-acetyl-glucosamine, amygdalin, glycogen, xylitol	+	–	+
Maltose	–	+	w
D-Tagatose, D-fucose, L-fucose	–	w	w
Major fatty acids	$C_{18:1}$, $C_{16:0}$, $C_{18:2}$	$C_{18:1}$, $C_{16:0}$, $C_{14:0}$	$C_{18:1}$, $C_{16:0}$, $C_{18:2}$
Polar lipids	PG, PC, UL, PL, AL	PG, PE, PC, UL, AL, PL*	PE, PG, PC, AL†
Ubiquinone	Q-10	Q-10, Q-9*	Q-10†
DNA G+C content (mol%)	50.0	52.4*	50.4†

All data are from this study unless otherwise indicated by *Teramoto and Nishijima [1] and †Nedashkovskaya *et al.* [2].

Table 2. Cellular fatty acid composition of strain 4G11^T, *Amylibacter marinus* NBRC 110140^T and *Amylibacter ulvae* KCTC 32465^T

Strains: 1, 4G11^T; 2, *Amylibacter marinus* NBRC 11014^T; 3, *Amylibacter ulvae* KCTC 32465^T. Fatty acids that represented <0.5 % in all strains were omitted. Major fatty acids are shown in bold type. –, Not detected. All data are from this study.

Fatty acid	1	2	3
$C_{12:1}$	1.6	1.5	2.0
$C_{14:0}$	–	5.0	–
$C_{16:0}$	8.7	5.3	6.5
$C_{18:0}$	1.1	–	1.4
$C_{18:1}$	81.9	85.3	86.8
$C_{18:2}$	6.7	2.8	3.4

pH 12, the presence of α-glucosidase activity, the ability to ferment sucrose as well as the inability to ferment maltose, D-tagatose, D-fucose, L-fucose and sorbose. The composition of the major fatty acids of strain 4G11^T was similar to those of reference type strains from the genus *Amylibacter* but the relative proportions of certain fatty acids were different. On the basis of phylogenetic, phenotypic and chemotaxonomic differences between strain 4G11^T and the other members of the genus *Amylibacter*, we suggest that strain 4G11^T represents a novel species of the genus *Amylibacter*, for which the name *Amylibacter kogurei* sp. nov. is proposed.

DESCRIPTION OF *AMYLIBACTER KOGUREI* SP. NOV.

Amylibacter kogurei (ko.gu′re.i. N.L. gen. n. *kogurei* of Kogure, to honour the Japanese microbiologist, Kazuhiro

Kogure in recognition of his contribution to the field of marine microbiology).

Cells of strain 4G11^T are rod-shaped, 1.39±0.39 µm (range, 0.76–2.47 µm) in length and 0.49±0.08 µm (0.39–0.70 µm) in diameter. Colonies grown on 1/2 MA after 3 days are white in colour, circular with entire edges. Strain 4G11^T is strictly aerobic and Gram-negative. The growth conditions for strain 4G11^T are 5–30 °C (optimum, 15–30 °C), 1–7 % NaCl (w/v) (5–7 %) and pH 4–12 (pH 7–10). Catalase- and oxidase-positive. DNA, aesculin, starch and urea are hydrolysed but chitin, CM-cellulose and gelatin are not. Acetoin is produced but not indole. Strain 4G11^T is not able to reduce nitrate to nitrite. For the API ZYM test, results for alkaline phosphate, esterase lipase, esterase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase are positive, but those for trypsin, α-chymotrypsin, acid phosphate, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Acid is produced from L-arabinose, ribose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, methyl α-D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol and 5-keto-gluconate in API 50CH. Positive results for β-galactosidase (PNPG) were obtained with API20NE and β-galactosidase (ONPG) and acid production from arabinose were observed in API20E. The G+C content of the genomic DNA of strain 4G11^T is 50.0 mol% and the only isoprenoid quinone is Q-10. The major cellular fatty acids of strain 4G11^T are $C_{18:1}$, $C_{16:0}$ and $C_{18:2}$. The major polar lipids for strain 4G11^T are phosphatidylglycerol, phosphatidylcholine, one unidentified polar lipid, one unidentified phospholipid and one unidentified aminolipid. The minor polar lipids are three unidentified polar lipids (UL2–4).

The type strain is 4G11^T (KY463497=KCTC 52506^T=NBRC 112428^T), which was isolated from the sea surface microlayer of Aburatsubo Inlet in Misaki, Kanagawa Prefecture, Japan.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Teramoto M, Nishijima M. *Amylibacter marinus* gen. nov., sp. nov., isolated from surface seawater. *Int J Syst Evol Microbiol* 2014;64:4016–4020.
2. Nedashkovskaya OI, Kukhlevskiy AD, Zhukova NV, Kim SB. *Amylibacter ulvae* sp. nov., a new alphaproteobacterium isolated from the Pacific green alga *Ulva fenestrata*. *Arch Microbiol* 2016;198:251–256.
3. Wang D, Wei Y, Cui Q, Li W. *Amylibacter cionae* sp. nov., isolated from the sea squirt *Ciona savignyi*. *Int J Syst Evol Microbiol* 2017;67:3462–3466.
4. Liss PS, Duce RA. *The Sea Surface and Global Change*. Cambridge: Cambridge University Press; 2005.
5. Franklin MP, McDonald IR, Bourne DG, Owens NJ, Upstill-Goddard RC *et al.* Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. *Environ Microbiol* 2005;7:723–736.
6. Cunliffe M, Schäfer H, Harrison E, Cleave S, Upstill-Goddard R *et al.* Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary. *ISME J* 2008;2:776–789.
7. Harvey GW. Microlayer collection from the sea surface: a new method and initial results. *Limnol Oceanogr* 1966;11:608–613.
8. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M (editors). *Nucleic Acid Techniques in Bacterial Systematics*. Chichester: Wiley; 1991.
9. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–410.
10. Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. *Nucleic Acids Res* 2016;44:D67–D72.
11. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y *et al.* Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
12. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.
13. Kimura M. *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press; 1983.
14. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
15. Felsenstein J. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981;17:368–376.
16. Fitch WM. Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* 1987;20:406–416.
17. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
18. Ezaki T, Hashimoto Y, Yamamoto H, Lucida ML, Liu SL *et al.* Evaluation of the microplate hybridization method for rapid identification of *Legionella* species. *Eur J Clin Microbiol Infect Dis* 1990;9:213–217.
19. Børsheim KY, Bratbak G, Heldal M. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Appl Environ Microbiol* 1990;56:352–356.
20. Harshey RM. Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* 2003;57:249–273.
21. Atlas RM. In: Parks LC (editor). *Handbook of Microbiological Media*. Boca Raton, FL: CRC Press; 1993.
22. Bowman JP. Description of *Cellulophaga algicola* sp. nov., isolated from the surfaces of Antarctic algae, and reclassification of *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Cellulophaga uliginosa* comb. nov. *Int J Syst Evol Microbiol* 2000;50:1861–1868.
23. Sakazaki R, Miki K, Yoshizaki E. *New Bacterial Culture Media*. Tokyo, Japan: Kindai Shuppan; 1995.
24. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–917.
25. Minnikin DE, Patel PV, Alshamaony L, Goodfellow M. Polar lipid composition in the classification of *Nocardia* and related bacteria. *Int J Syst Bacteriol* 1977;27:104–117.
26. Kawamura Y. Methods for bacterial classification and identification. The textbook for the 18th technical training course of the Japanese Society for Bacteriology. *Jpn Soc Bacteriol* 2000;55:545–584.

27. **Suzuki K.** DNA-DNA hybridization. In: Suzuki K, Hiraishi A and Yokota A (editors). *Experimental Techniques for the Classification and Identification of Microorganisms*. Tokyo: Springer-Verlag; 2001.
28. **Katayama-Fujimura Y, Komatsu Y, Kuraishi H, Kaneko T.** Estimation of DNA base composition by high performance liquid chromatography of its nuclease P1 hydrolysate. *Agric Biol Chem* 1984;48: 3169–3172.
29. **Wong SK, Yoshizawa S, Nakajima Y, Ogura Y, Hayashi T *et al.*** Draft genome sequence of *Lewinella* sp. strain 4G2 isolated from the coastal sea surface microlayer. *Genome Announc* 2016;4: e00754-16.
30. **Kajitani R, Toshimoto K, Noguchi H, Toyoda A, Ogura Y *et al.*** Efficient *de novo* assembly of highly heterozygous genomes from whole-genome shotgun short reads. *Genome Res* 2014;24:1384–1395.
31. **Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Ciufo S *et al.*** Prokaryotic genome annotation pipeline. In: *The NCBI Handbook*, 2nd ed. NCBI, Bethesda: MD; 2013.