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Characterization of *Lacinutrix neustonica* sp. nov., Isolated from the Sea Surface Microlayer of Brackish Lake Shihwa, South Korea

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Abstract: A Gram-negative, strictly aerobic, non-motile, slightly curved rod-shaped bacterial strain, designated as HL-RS19^T, was isolated from a sea surface microlayer (SML) sample of the brackish Lake Shihwa. Here, we characterized the new strain HL-RS19^T using a polyphasic approach to determine its taxonomic position. A phylogenetic analysis of its 16S rRNA gene sequence revealed that strain HL-RS19^T belonged to the genus *Lacinutrix* and was closely related to *L. mariniflava* AKS432^T (97.9%), *L. algicola* AKS293^T (97.8%), and other *Lacinutrix* species (<97.3%). The complete genome sequence of strain HL-RS19^T comprised a circular chromosome of 3.9 Mbp with a DNA G+C content of 35.2%. Genomic comparisons based on the average nucleotide identity and digital DNA-DNA hybridization showed that strain HL-RS19^T was consistently discriminated from its closely related taxa in the genus *Lacinutrix*. Strain HL-RS19^T showed optimal growth at 20–25 °C, pH 6.5–7.0, and 3.0–3.5% (*w*/*v*) sea salts. The major fatty acids (>5%) of strain HL-RS19^T were identified as iso-$C_{15:1}$ G (16.5%), iso-$C_{16:0}$ 3-OH (12.9%), anteiso-$C_{15:1}$ A (9.9%), anteiso-$C_{15:0}$ (9.7%), iso-$C_{15:0}$ (9.0%), and iso-$C_{15:0}$ 3-OH (8.3%). The polar lipids consisted of phosphatidylethanolamine, three unidentified aminolipids, an unidentified phospholipid, and two unidentified lipids. The major respiratory quinone was MK-6. Based on phylogenetic, genomic, phenotypic, and chemotaxonomic data, strain HL-RS19^T represents a novel species belonging to the genus *Lacinutrix*, for which the name *Lacinutrix neustonica* sp. nov. is proposed. The type strain is HL-RS19^T (=KCCM 90497^T = JCM 35710^T). The genome sequence analysis of strain HL-RS19^T suggests that it may be well adapted to a harsh SML environment and is likely involved in arsenic cycling, potentially contributing to the bioremediation of anthropogenic arsenic pollution.

Keywords: *Lacinutrix neustonica* sp. nov.; Lake Shihwa; neuston; new species; sea surface microlayer

Citation: Choi, J.Y.; Kim, S.Y.; Hong, Y.W.; Kim, B.J.; Shin, D.Y.; Kang, J.K.; Cho, B.C.; Hwang, C.Y. Characterization of *Lacinutrix neustonica* sp. nov., Isolated from the Sea Surface Microlayer of Brackish Lake Shihwa, South Korea. *Diversity* **2023**, *15*, 1004. https://doi.org/10.3390/d15091004

Academic Editors: Massimiliano Fenice, Susanna Gorrasi, Marcella Pasqualetti and Ipek Kurtboke

Received: 14 July 2023
Revised: 28 August 2023
Accepted: 6 September 2023
Published: 9 September 2023

1. Introduction

The sea surface microlayer (SML) represents the uppermost boundary layer of the ocean, where the exchange of gases and particles takes place between the ocean and the atmosphere [1,2]. With a total thickness ranging from 1 to 1000 µm, the SML exhibits distinct physicochemical and biological characteristics compared to the underlying water (UW) [3,4]. The unique characteristics of the SML can be attributed to several factors, including strong ultraviolet (UV) irradiation, the high activity of photoreactions, and the intense accumulation of autochthonous/allochthonous organic or inorganic matter [5,6]. Previous studies have reported the significant enrichment of anthropogenic pollutants, such as hydrocarbon compounds and heavy metals, in the coastal SML near urban, agricultural, and industrial areas [1,5,7–9]. Therefore, the bacterial inhabitants of the SML, known

as bacterioneuston, present an intriguing natural model for studying the restoration of damages caused by UV radiation or radical oxidants, as well as for obtaining valuable biological resources for the bioremediation of toxic substances. In fact, a new bacterial species in diverse lineages has been successfully isolated from SML samples collected from pristine to industrial environments [10–15].

The genus *Lacinutrix* was first described alongside *Lacinutrix copepodicola* [16], and it currently belongs to the family *Flavobacteriaceae* in the phylum *Bacteroidota*. At the time of writing, twelve species of *Lacinutrix* have been reported, with validly published names [17]. Members of the genus *Lacinutrix* are known to be neutrophilic, Gram-negative, non-motile or motile by gliding, strictly aerobic, straight, slightly rod-shaped, or coccoid cells that are catalase- and oxidase-positive and that produce golden-yellow, orange, or orange-yellow pigments [18–20]. The type strains of the genus *Lacinutrix* have been isolated from diverse marine habitats, such as seawater, sediment, macroalgae, copepod, clam, crab, and flounder, from temperate to polar environments [16,19–28]. During a year-round investigation of the SML at a temperate coast near an industrial complex, we isolated a neustonic bacterium identified as belonging to the genus *Lacinutrix* and designated as HL-RS19^T. In this study, we conducted a comprehensive characterization of strain HL-RS19^T, including its phylogenetic, genomic, phenotypic, and chemotaxonomic properties to accurately position it within the genus *Lacinutrix* in terms of taxonomic classification.

2. Materials and Methods

2.1. Habitat, Isolation and Cultivation Conditions

The SML sample was collected using a glass plate sampler [4] in the brackish Lake Shihwa (37°18′3.6″ N, 126°43′58.8″ E), South Korea, in January 2020. This research area receives the riverine discharge from a nearby industrial complex. During the sampling, the water temperature and salinity were 3 °C and 36.3 ‰, respectively. For cultivation, an aliquot (100 µL) of the SML sample was spread onto a saline Reasoner's 2A agar (R2A; BD Difco, Franklin Lakes, NJ, USA) medium supplemented with 3% (*w*/*v*) sea salts (Sigma-Aldrich, St. Louis, MO, USA). The saline R2A medium was incubated aerobically at 25 °C for 4 days. Strain HL-RS19^T was isolated and purified via subculturing more than four times. In a preliminary test, strain HL-RS19^T grew better on the marine agar (MA; BD Difco, Franklin Lakes, NJ, USA) than on the saline R2A agar. After, strain HL-RS19^T was routinely cultivated on an MA at 25 °C. The strain was preserved in a marine broth (MB; BD Difco, Franklin Lakes, NJ, USA) supplemented with 20 % (*v*/*v*) glycerol and stored at −70 °C. Over the course of four seasonal surveys conducted throughout a year in the study area, 212 bacterial strains were isolated from SML samples, and identified by the sequencing the 16S rRNA genes, as detailed in Section 2.2. Among them, strain HL-RS19^T was discovered as a singular occurrence in the genus *Lacinutrix*, appearing only once during the entire sampling period.

2.2. 16S rRNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA was extracted from a single colony using the boiling method as previously described [29]. The 16S rRNA gene of the strain HL-RS19^T was amplified through polymerase chain reaction (PCR) utilizing the universal primers 27F and 1492R [30]. Subsequently, the PCR product was subjected to purification using ExoSAP-IT (ThermoFisher Scientific, Waltham, MA, USA). Direct sequencing of the purified PCR products was performed using four sequencing primers—27F, 337F, 907R, and 1492R [30,31]—via an Applied Biosystems sequencer (ABI 3730XL) at Cosmo Genetech (Seoul, Republic of Korea). Almost the full length of the 16S rRNA gene sequence of strain HL-RS19^T (1419 bp) was assembled with CodonCode Aligner version 10.0.2 (CodonCode Co., Centerville, OH, USA) and analyzed using GenBank BLAST searches and EzBioCloud databases [32]. The validly published phylogenetic neighbors and the complete 16S rRNA gene sequences of strain HL-RS19^T were aligned using the EzEditor2 [33] considering the secondary structure of the bacterial 16S rRNA. Subsequently, the phylogenetic analyses were performed using MEGA

version 11 [34]. A neighbor-joining (NJ) tree [35] was reconstructed using the Jukes-Cantor model [36] with uniform rates and pairwise deletion options. A maximum-likelihood (ML) tree [37] was reconstructed using the Kimura two-parameter model [38] with gamma distributed with invariant sites (G+I), using all sites option for gaps/missing data. A maximum-parsimony (MP) tree [39] was constructed using the Subtree-Pruning-Regrafting (SPR) search method [40] with the number of initial trees (random addition) as 10, using all sites options. The robustness of the phylogenetic trees was evaluated by performing a bootstrap analysis based on 1000 replicates [41].

2.3. Genome Sequencing, Assembly, Annotation, and Phylogenomic Analysis

For genome comparison, the genomic DNA of strain HL-RS19^T was extracted following the manufacturer's protocol using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The whole genome sequencing was performed using a sequencing library for the MinION Mk1C sequencer (Oxford Nanopore Technologies; ONT, Oxford, UK) with a native barcoding expansion kit 13-24 (EXP-NBD114; ONT, Oxford, UK) and the ligation sequencing kit (SQK-LSK109; ONT, Oxford, UK). Raw reads were base-called, demultiplexed, and adapter-trimmed using MinKNOW version 22.05.6 and Guppy version 6.3.8. De novo genome assembly was performed using Flye version 2.9.1 [42] and polished using Medaka version 1.7.2 https://github.com/nanoporetech/medaka (accessed on 21 November 2022). The genome size, N50, and DNA G+C content were calculated using QUAST version 5.2.0 [43]. Genome coverage was measured using SAMtools version 1.11 [44]. The overall genome relatedness index (OGRI) values, including average nucleotide identity (ANI) [45] and digital DNA-DNA hybridization (dDDH) by the genome-to-genome distance calculator (GGDC) [46], were obtained for all pairwise comparisons. Additionally, a complete 16S rRNA gene sequence was retrieved from the genome sequence of strain HL-RS19^T using the ContEst16S program [47].

A phylogenomic analysis of strain HL-RS19^T and the type strains of related species was performed based on the Genome Taxonomy Database (GTDB) taxonomy using GTDB-Tk [48]. The genome of strain HL-RS19^T was compared with the type strains of related species, including six validly named *Lacinutrix* species and other taxonomically differentiated *Flavobacteriaceae* family members, which were available in the NCBI Genome database (Table S1). The amino acid sequences of 120 concatenated marker genes from the genomes were detected and aligned using the GTDB-Tk tool. Phylogenomic trees were reconstructed using the NJ, MP, and ML methods, with 1000 replications of the bootstrap analysis using MEGA version 11 [34].

A genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [49]. The Metabolic Pathway/Genome Database (PGDB) was generated computationally using the PathoLogic component of Pathway Tools software version 26.0 and MetaCyc version 23.0 [50,51]. A search for the biosynthetic gene clusters of secondary metabolites was performed using antiSMASH version 6.1.1 [52] with the option of strict detection. For a comparative genomic investigation, genome sequences of *Lacinutrix* available in the NCBI Genome database were retrieved and analyzed to search for protein-coding genes using tBLASTn version 2.9.0 with an e-value threshold of $1e^{-5}$. The genomic structure and variations within the *Lacinutrix* genus were evaluated by utilizing the Integrated Prokaryotes Genome and Pan-genome Analysis (IPGA) web server https://nmdc.cn/ipga (accessed on 25 May 2023) [53] with default settings.

2.4. Physiological, Morphological and Biochemical Characteristics

Based on its 16S rRNA gene sequence, *Lacinutrix mariniflava* AKS432^T (=KCCM 42306^T) [25], which was most similar to strain HL-RS19^T by 97.9%, was purchased from the Korean Culture Center of Microorganisms (KCCM) and used as a reference strain. Unless otherwise specified, *L. mariniflava* KCCM 42306^T and strain HL-RS19^T were incubated on an MA for 3–4 days at 20 °C and 25 °C, respectively, for phenotypic tests under the exponential growth phase. According to the minimal standards for describing a new taxon

of the family *Flavobacteriaceae* [54], all experiments for the physiological characteristics of strain HL-RS19^T were carried out in duplicates, along with the type strain of *L. mariniflava* KCCM 42306^T.

The temperature range for growth was tested on an MA at 5–40 °C (5 °C intervals) for 2 weeks. A salt-tolerance test was carried out using synthetic ZoBell broth (Bacto peptone 5 g, yeast extract 1 g, and ferric citrate 0.1 g per liter of distilled water) [55] supplemented with 0–4% (0.5% intervals), 5–10% (1% intervals), 12%, and 15% (*w*/*v*) of sea salts (Sigma-Aldrich, St. Louis, MO, USA). The growth under different pH values (pH 5.0–10.0) was investigated via inoculation in MB adjusted using pH buffer systems (MES, pH 5.0–6.5; MOPS, pH 7.0–7.5; AMPD, pH 8.0–9.5; CAPS, pH 10.0). Growth was monitored by measuring the optical density at 600 nm (SPECTRostar Nano spectrophotometer, BMG Labtech, Ortenberg, Germany) at 1–3 days intervals for 2 weeks. Anaerobic growth was assessed on both the MA and MA supplemented with potassium nitrate (0.1%, *w*/*v*) as an electron acceptor [56] and incubated in an anaerobic jar with AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) for 2 weeks. Gram-staining was performed using a Gram-Staining kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Motility was tested using a semi-solid MA (0.4% agar, *w*/*v*) method [57] and the hanging drop method [54,58]. Cell morphology and size were examined via transmission electron microscopy (LIBRA 120; Carl Zeiss, Oberkochen, Germany) using the strain grown on an MA at 25 °C for 3 days. The presence of carotenoid for the cells incubated in both light and dark conditions for 3 days was examined using the spectrophotometric test, as previously described [59]. Flexirubin-type pigments were determined using two different methods, as previously described [59,60].

In addition, the biochemical test and enzyme activities of strain HL-RS19^T and *L. mariniflava* KCCM 42306^T were determined by using API 20E, API 20NE, and API ZYM kits (bioMérieux) according to the manufacturer's recommendations, except that the cells were resuspended in distilled water containing 3% (*w*/*v*) sea salts and were incubated at the optimal growth temperature for each bacterial strain. Catalase and oxidase tests were performed as previously described [61]. The hydrolysis of starch; Tweens 40, 60, 80; casein; gelatin; and urea was tested on an MA supplemented with the corresponding substrates [62]. The hydrolysis of xanthine, hypoxanthine, and L-tyrosine was examined using the method as described [63]. The hydrolysis of aesculin and the nitrate reduction test was determined by the method as described [64]. The ability to utilize a sole carbon source was tested by inoculating bacterial suspension into the basal medium consisting of NaCl 2.36 g, KCl 0.06 g, $MgCl_2 \cdot 6H_2O$ 4.5 g, $MgSO_4 \cdot 7H_2O$ 5.9 g, $CaCl_2 \cdot 2H_2O$ 1.3 g, $NaNO_3$ 0.2 g, NH_4Cl 0.2 g, and yeast extract 0.05 g per liter of distilled water, supplemented with a final concentration of 0.4% (*w*/*w*) tested carbon sources [11]. Carbon utilization was determined as being negative when the growth was equal to or less than that in the negative control without a carbon source. The growth was determined by monitoring changes in the OD_{600} for 3 weeks.

The chemotaxonomic characteristics of strain HL-RS19^T and *L. mariniflava* KCCM 42306^T were determined using the cells grown on the MA for 3 days at 20 °C. The cellular fatty acid composition was analyzed via gas chromatography based on the Microbial Identification System (MIDI, Microbial ID) and RTSBA 6 version 6.21 at the KCCM. The polar lipids of strain HL-RS19^T and *L. mariniflava* KCCM 42306^T were identified via thin-layer chromatography (TLC) followed by spraying with appropriate detection reagents [65,66] at the KCCM. The isoprenoid quinone composition of strain HL-RS19^T was determined as previously described [66,67] and analyzed via HPLC at the KCCM.

3. Results and Discussion

3.1. 16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA gene sequence of strain HL-RS19^T (1419 bp), determined via direct sequencing, was almost identical (99.8–99.9%) to the three copies of the complete 16S rRNA gene sequences (1518 bp) retrieved from the genome sequence of strain HL-RS19^T. At the

16S rRNA gene sequence level, strain HL-RS19^T was most closely related to *L. mariniflava* AKS432^T with a similarity of 97.9% and followed by *Lacinutrix algicola* AKS293^T with a similarity of 97.8%. The 16S rRNA gene similarity values between strain HL-RS19^T and other type strains of *Lacinutrix* species were below 97.3%. The phylogenetic analyses of the 16S rRNA gene sequences revealed that strain HL-RS19^T formed a robust clade with *L. mariniflava* AKS432^T and *L. algicola* AKS293^T, which was consistently recovered in all phylogenetic trees (NJ, ML, and MP) (Figure 1 and Figure S1). Therefore, the phylogenetic position of strain HL-RS19^T showed that the strain could be assigned to a novel species in the genus *Lacinutrix*.

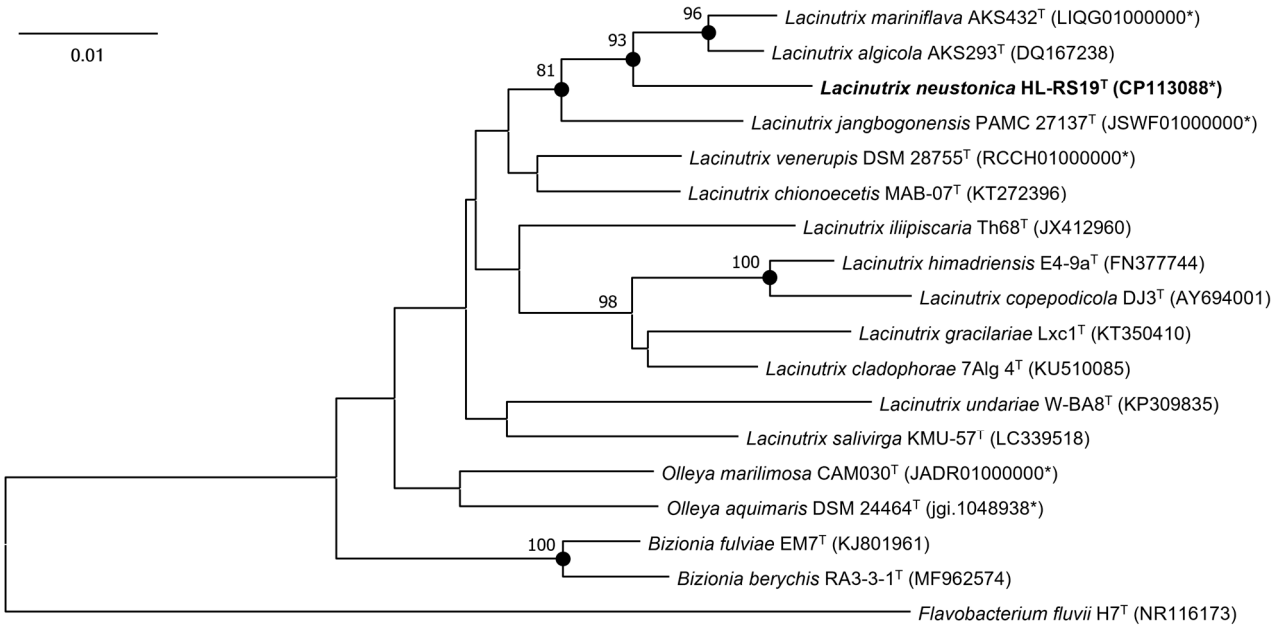

Figure 1. Neighbor-joining tree based on the 16S rRNA gene sequences of HL-RS19^T and related taxa in the family *Flavobacteriaceae*. *Flavobacterium fluvii* H7^T was used as an outgroup. Bootstrap values at nodes indicate a percentage higher than 70% (based on 1000 replicates). Filled circles indicate that the corresponding nodes were recovered in the maximum-likelihood and the maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position. The asterisk mark in the parentheses indicates 16S rRNA gene sequence retrieved from the genome sequence of the type strain.

3.2. Genome Analysis and Genomic Features

The complete genome size of strain HL-RS19^T was 3.9 Mbp, with a DNA G+C content of 35.2 mol% (Table S1). The ANI values between strain HL-RS19^T and the closely related *Lacinutrix* species (i.e., *L. mariniflava* AKS432^T and *L. algicola* AKS293^T) were 75.2–75.3% (Table 1). The genomic relatedness analysis based on genome-to-genome distance showed that HL-RS19^T was related to *L. mariniflava* AKS432^T and *L. algicola* AKS293^T via dDDH values of 19.7% and 20.2%, respectively (Table 1). This level is obviously below the proposed ANI and dDDH cut-off values (95–96% and 70%, respectively) for delineating bacterial species [45,68]. These results indicate that strain HL-RS19^T is a new member of a distinct species of the genus *Lacinutrix*.

In the phylogenomic tree, strain HL-RS19^T formed a discriminated clade with *L. algicola* AKS293^T, *L. mariniflava* AKS432^T, *L. jangbogonensis* PAMC 27137^T, and *L. venerupis* DSM 28755^T (Figures 2 and S2), which were differed in the phylogenetic trees of the 16S rRNA gene sequences (Figure 1), resulting in somewhat different tree topologies. An incongruity between 16S rRNA gene- and genome-based trees in the genus *Lacinutrix* was found in this study.

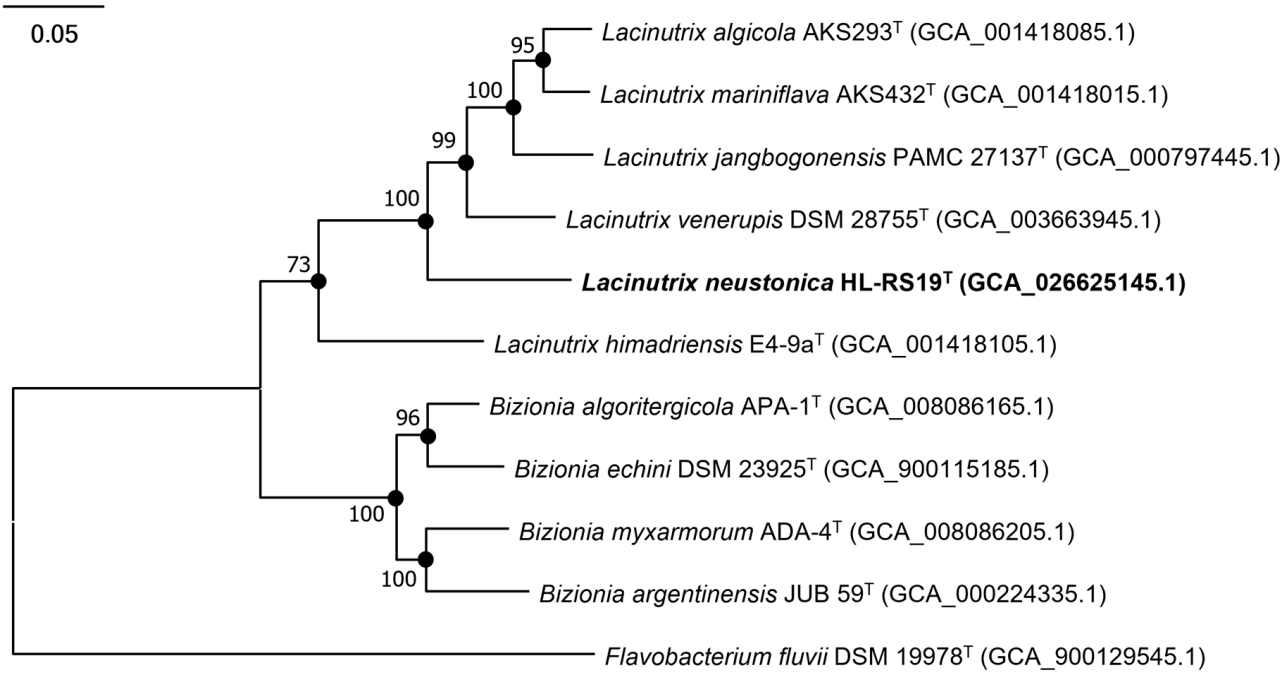

Figure 2. Maximum-likelihood tree based on the amino acid sequences for 120 concatenated marker genes of strain HL-RS19^T and related taxa in the family *Flavobacteriaceae*. *Flavobacterium fluvii* H7^T was used as an outgroup. Bootstrap values at nodes indicate a percentage higher than 70% (based on 1000 replicates). Filled circles indicate that the corresponding nodes were recovered in the neighbor-joining and the maximum-parsimony trees. Bar, 0.05 substitutions per amino acid position.

Table 1. Results of genomic relatedness analyses based on the average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values. 1, *Lacinutrix neustonica* HL-RS19^T (GCA_026625145.1); 2, *Lacinutrix mariniflava* KCCM 42306^T (GCA_001418015.1); 3, *Lacinutrix algicola* AKS293^T (GCA_001418085.1); 4, *Lacinutrix jangbogonensis* PAMC 27137^T (GCA_000797445.1); 5, *Lacinutrix venerupis* DSM 28755^T (GCA_003663945.1); 6, *Lacinutrix himadriensis* E4-9a^T (GCA_001418105.1). The ANI values are indicated in the region above the diagonal grey area (values of 100%). The dDDH values are depicted below the diagonal grey area (values of 100%).

	ANI/dDDH Value (%)					
dDDH\ANI	**1**	**2**	**3**	**4**	**5**	**6**
1	–	75.2	75.3	75.5	75.3	73.5
2	19.7	–	86.5	82.0	80.6	75.8
3	20.2	31.8	–	81.8	80.7	76.0
4	20.1	25.9	25.6	–	80.2	75.4
5	19.6	23.5	23.7	23.4	–	75.5
6	19.8	21.2	21.5	20.6	19.9	–

The genomic analyses revealed that strain HL-RS19^T possesses genes that might increase its fitness to a harsh SML environment at a coastal industrial complex, such as strong UV irradiation and the relative enrichment of the heavy metals from anthropogenic and/or natural sources [4,5]. A carotenoid biosynthetic gene cluster (BGC) was detected in the genome sequence of strain HL-RS19^T (Figure 3), and the production of a carotenoid pigment was experimentally confirmed in the present study (Figure S3). Carotenoid pigments are known to be effective in UV absorption and screening in heterotrophic bacteria [69,70].

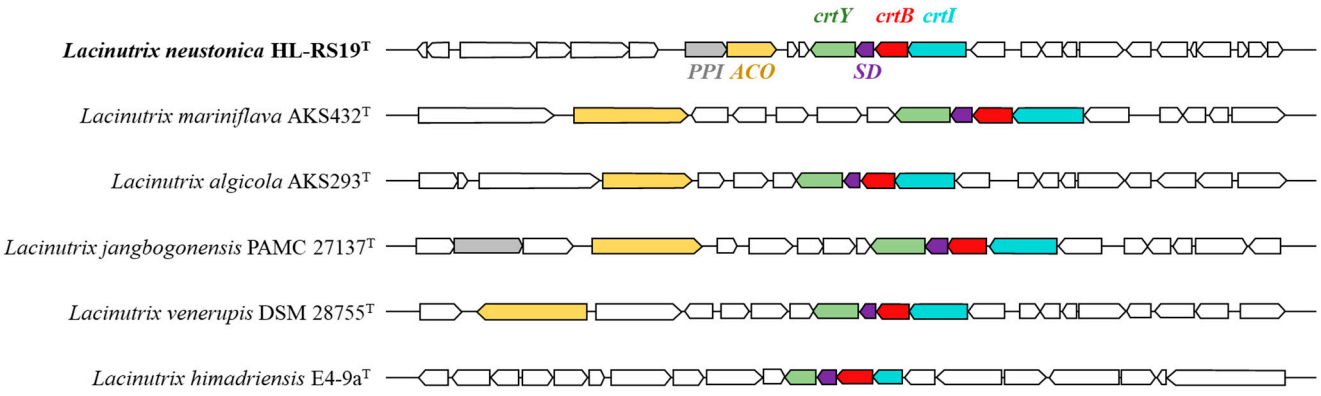

Figure 3. Schematic biosynthesis gene cluster of carotenoids predicted from the genome sequence of *Lacinutrix* type strains identified using antiSMASH [52]. Each color represents the corresponding gene. PPI, peptidylprolyl isomerase (grey); ACO, aconitate hydratase (yellow); *crtY*, lycopene cyclase (green); SD, sterol desaturase (purple); *crtB*, phytoene/squalene synthase (red); *crtI*, phytoene desaturase (light blue).

In addition, the carotenoid biosynthetic gene cluster was identified in all *Lacinutrix* species through the analysis of genomic sequences using tBLASTn (Figure 3). The genus *Lacinutrix* was speculated to possess the capability to biosynthesize β-carotene through the utilization of key genetic elements, namely phytoene synthase (*crtB*), phytoene desaturase (*crtI*), and lycopene cyclase (*crtY*). The initial step in carotenoid production involves the enzymatic conversion of the immediate precursor, geranylgeranyl diphosphate (GGPP) [71], into phytoene via the activity of *crtB* [72]. Subsequently, the *crtI* gene facilitates the synthesis of lycopene from phytoene [72], and ultimately, the *crtY* gene governs the conversion of lycopene into β-carotene [72]. Although certain *Lacinutrix* species may lack specific components of the carotenoid gene cluster, the majority of them possess the essential *crtB*, *crtI*, and *crtY* genes. The presence of these carotenoid biosynthetic genes in their genomes indicates the presumed production of β-carotene as the ultimate product of the biosynthetic pathway.

Multiple DNA repair systems to restore UV-induced DNA damage were found in strain HL-RS19^T, including light-dependent photoreactivation (DNA photolyase), nucleotide excision repair (*UvrABC* excinuclease complex), and homologous recombination repair (Holliday junction helicase complex) [73,74]. To cope with arsenic-rich conditions in an SML environment, strain HL-RS19^T has an arsenic detoxification system, which comprises essential genes encoding *ArsR* transcriptional regulator, arsenate reductase (*ArsC*), and arsenite efflux transport protein *ArsB* [75], suggestive of its participation in the biogeochemical cycling of arsenic in such an environment. In practical applications, arsenic-resistant bacteria have been utilized for bioremediation as effective agents for reducing harmful metal concentrations [76–79]. The existence of the *ars* operon (*arsRCB*) in strain HL-RS19^T potentially signifies its capability as a promising bioremediation tool [76], whether deployed as a wild-type strain or as genetically engineered microbes, to address environmental arsenic contamination across a broad spectrum of salinity levels.

The orthologous genes present in six genomes of *Lacinutrix* species were systematically classified into core, accessory, and unique gene clusters using IPGA (Figure 4). Among these, 8.4% were classified as core gene clusters, indicating their presence across all genomes, while 70.8% were classified as unique gene clusters specific to particular genes in different genomes (Figure 4). Notably, strain HL-RS19^T exhibited the highest number of unique gene clusters (3214) among all currently known species within the genus *Lacinutrix*. Most of the unique genes were classified as unknown, with only 263 genes (8%) being annotated. Specifically, genes associated with antimicrobial resistance (*tetM* and *catB*), restriction and modification system (*hsdS*, *yhdJ*, and *mcrC*), and DNA repair and recombination proteins (*uvrA*, *uvrC*, *recN*, and *gyrB*) are present in strain HL-RS19^T.

Figure 4. Pan-genome profile of *Lacinutrix* species. Orthologous genes of individual genomes were clustered into three groups: core genes, accessory genes and unique genes. CP, genome completeness; SN, scaffold number; GC, GC content; GN, gene number; GL, genome length.

3.3. Physiological, Morphological and Biochemical Characteristics

Strain HL-RS19^T grew at 10–30 °C, with its optimum at 20–25 °C (Table 2). In addition, strain HL-RS19^T was able to grow at pH 6.0–8.5, with its optimum at pH 6.5–7.0. Strain HL-RS19^T required salt to grow and was tolerant up to 7.0% (*w*/*v*) (Table 2). Strain HL-RS19^T was strictly aerobic, Gram-negative, non-motile, and slightly curved rod-shaped cells (Figure 5) that displayed activities of catalase and oxidase, and were capable of carotenoid formation, which are common properties of the genus *Lacinutrix* [18–20]. Its other physiological and biochemical characteristics are summarized in Table 2, along with the species description.

The major fatty acids (>5%) of strain HL-RS19^T were iso-$C_{15:1}$ G (16.5%), iso-$C_{16:0}$ 3-OH (12.9%), anteiso-$C_{15:1}$ A (9.9%), anteiso-$C_{15:0}$ (9.7%), iso-$C_{15:0}$ (9.0%), and iso-$C_{15:0}$ 3-OH (8.3%); a detailed fatty acid composition is given in Table S2. The fatty acid profile of strain HL-RS19^T was very similar to that of *L. mariniflava* KCCM 42306^T (Table S2), except some minor fatty acids (cyclopropane fatty acids and some unsaturated fatty acids) were not detected in strain HL-RS19^T. The polar lipids of strain HL-RS19^T were phosphatidylethanolamine (PE), three unidentified aminolipids (AL1–3), an unidentified phospholipid (PL), and two unidentified lipids (L1–2), which were nearly identical to those of *L. mariniflava* KCCM 42306^T, except for the absence of an unidentified lipid (L3) (Figure S4). MK-6 was identified as the only menaquinone present in strain HL-RS19^T, which is the same as that found in other *Lacinutrix* spp. [16,19–22,24–28].

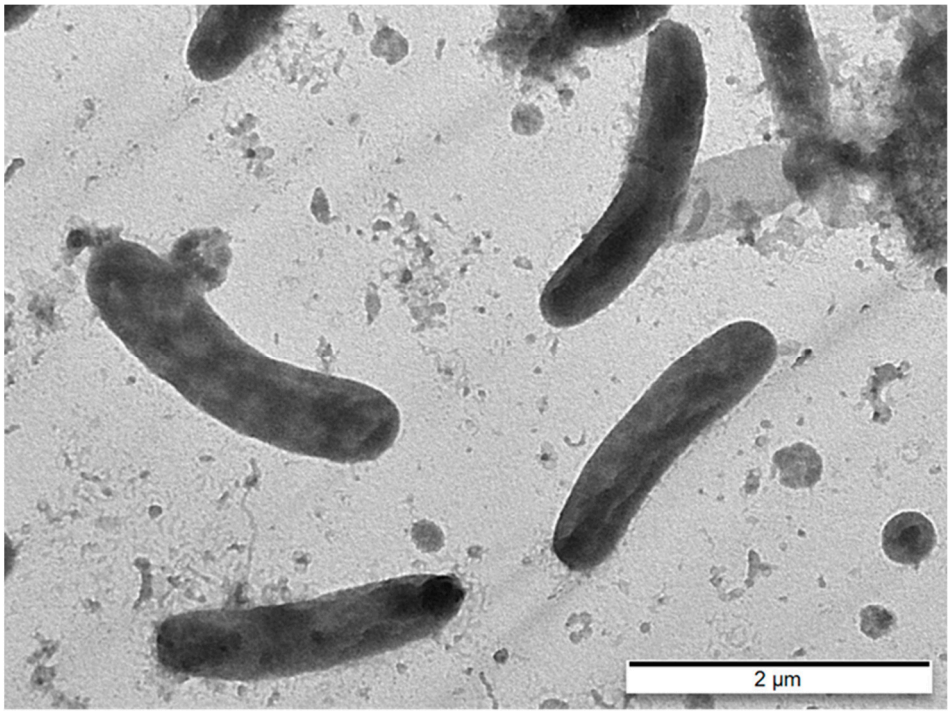

Figure 5. Transmission electron micrograph (TEM) of negatively stained cells of strain HL-RS19^T grown on marine agar at 25 °C for 3 days. Bar, 2.0 μm.

Table 2. Physiological characteristics that distinguish strain HL-RS19^T from other closely related species of the genus *Lacinutrix*. 1: *Lacinutrix neustonica* HL-RS19^T (this study), 2: *Lacinutrix mariniflava* KCCM 42306^T (this study), and 3: *Lacinutrix algicola* AKS293^T [25]. +, Positive; −, negative.

Characteristics	1	2	3
Growth temperature range (optimum; °C)	10–30 (20–25)	5–20 (15–20)	0–25 (17.5)
Salt tolerance range (optimum;%, *w*/*v*)	1.0–7.0 (3.0–3.5)	1.5–6.0 (2.5–3.0)	0–2.5 (0.5)
pH range (optimum)	6.0–8.5 (6.5–7.0)	6.0–8.5 (6.5–7.0)	5.5–8.5 (6.5)
Hydrolysis of:			
Casein	−	+	+
Gelatin	− *	+ *	+
Tween 40	−	+	−
Tween 80	+	+	−
Urea	−	−	+
Utilization as sole carbon source:			
N-Acetyl-D-glucosamine	−	−	+
D-Glucose	−	+	−
D-Maltose	+	+	−
Mannitol	−	−	+
D-Mannose	+	−	−
Enzymatic activity (API ZYM) of:			
β-Galactosidase	−	−	+
DNA G+C content (mol%)	35.2	34.7 **	37.0

* API 20E and API 20NE assays showed the congruent results. ** Data from Nedashkovskaya et al. [25].

Strain HL-RS19^T could be phenotypically differentiated from its most closely related phylogenetic neighbor *L. mariniflava* KCCM 42306^T as follows: The temperature range for the growth of strain HL-RS19^T (10–30 °C) was higher than that of *L. mariniflava* KCCM 42306^T (5–20 °C; Table 2). The salt tolerance range of strain HL-RS19^T (1.0–7.0%) was broader than that of *L. mariniflava* KCCM 42306^T (1.5–6.0%; Table 2). In addition, strain HL-RS19^T could not hydrolyze casein, gelatin, and Tween 40, which were different characteristics from those of *L. mariniflava* KCCM 42306^T (Table 2). Strain HL-RS19^T could also

be distinguished from its other phylogenetical relative *L. algicola* AKS293^T, for example, the inability to grow at 0–5 °C, the ability to grow in the presence of 3–7% (*w*/*v*) sea salts, the ability to hydrolyze Tween 80, the inability to hydrolyze urea, and the absence of β-galactosidase activity (Table 2).

4. Conclusions

Based on the phylogenetic, genomic, phenotypic, and chemotaxonomic characteristics described above, strain HL-RS19^T should be placed in the genus *Lacinutrix* as representing a novel species, for which the name *Lacinutrix neustonica* sp. nov. is proposed. The presence of a suite of essential genes encoding arsenic detoxification processes in the genome of strain HL-RS19^T displays its potential for bioremediation in arsenic-contaminated saline environments.

Description of *Lacinutrix neustonica* sp. nov.:

Lacinutrix neustonica (neus.to'ni.ca. N.L. fem. adj. *neustonica* pertaining to and living in the neuston).

Cells are strictly aerobic, Gram-negative, non-motile, and slightly curved rod-shaped (0.3–0.8 μm wide and 1.4–4.2 μm long. Colonies are circular, shiny, golden-yellow, and convex with entire margins after 7 days of incubation on MA plates. They are positive for oxidase and catalase activities. Growth occurs at 10–30 °C (optimum at 20–25 °C), at pH 6.0–8.5 (optimum at 6.5–7.0), and in the presence of sea salts with a concentration of 1.0–7.0% (*w*/*v*) (optimum 3.0–3.5%). Starch, aesculin, Tweens 60 and 80 are hydrolyzed, but casein, gelatin, hypoxanthine, xanthine, L-tyrosine, Tween 40, and urea are not. Nitrate is not reduced. Carotenoid pigments are produced. Flexirubin-type pigments are not produced. In the API ZYM system, they are positive for acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase, but negative for N-acetyl-β-glucosaminidase, α-fucosidase, α- and β-galactosidases, α- and β- glucosidases, β-glucuronidase, lipase (C14), and α-mannosidase. In the API 20E system, they are positive for the Voges-Proskauer test, but negative for arginine dihydrolase, β-galactosidase, gelatinase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, urease, tryptophan deaminase, and the production of hydrogen sulfide and indole. In the API 20NE system, they are positive for esculin hydrolysis and paranitrophenyl-β-D-galactopyranosidase (weakly), but negative for glucose fermentation, hydrolysis of L-arginine, gelatin, urea, indole production, and nitrate reduction. D-Maltose, D-mannose, L-proline, and trisodium citrate are utilized as sole carbon sources, but acetate, N-acetyl-D-glucosamine, L-arabinose, D-glucose, inositol, L-lysine, malic acid, mannitol, potassium gluconate, pyruvate, and raffinose are not utilized. The major fatty acids are iso-$C_{15:1}$ G, iso-$C_{16:0}$ 3-OH, anteiso-$C_{15:1}$ A, anteiso-$C_{15:0}$, iso-$C_{15:0}$, and iso-$C_{15:0}$ 3-OH. The polar lipids are phosphatidylethanolamine, three unidentified aminolipids, an unidentified phospholipid, and two unidentified lipids. The menaquinone present is MK-6.

The type strain HL-RS19^T (=KCCM 90497^T = JCM 35710^T) was isolated from the surface microlayer sample of brackish Lake Shihwa. The GenBank/EMBL/DBBJ accession numbers for the 16S rRNA gene sequence and the genome sequence of strain HL-RS19^T are MZ820004 and CP113088, respectively. The DNA G+C content is 35.2%, determined via genome analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15091004/s1, Figure S1: (a) Maximum-likelihood (ML) and (b) maximum-parsimony (MP) phylogenetic tree based on 16S rRNA gene sequences of strain HL-RS19^T and related taxa in the family *Flavobacteriaceae*; Figure S2: (a) Neighbor-joining (NJ) and (b) maximum-parsimony (MP) phylogenomic tree based on the amino acid sequences for 120 concatenated marker genes of strain HL-RS19^T and related taxa in the family *Flavobacteriaceae*; Figure S3: UV-VIS spectra of diverse carotenoids detected in strain HL-RS19^T incubated under light and dark conditions; Figure S4: Two-dimensional thin-layer chromatography (TLC) of the polar lipids of (a) *Lacinutrix neustonica* HL-RS19^T and (b) *Lacinutrix mariniflava* KCCM 42306^T; Table S1: List of whole

genome sequences of *Lacinutrix* spp.; Table S2: Cellular fatty acid composition (%) of strain HL-RS19^T and *L. mariniflava* KCCM 42306^T.

Author Contributions: Isolation, morphological, and biochemical characterization of strains, J.Y.C., S.Y.K. and Y.W.H.; chemotaxonomic characterization, S.Y.K.; Sanger sequencing, J.Y.C. and J.K.K.; genome sequencing, phylogenetic, and phylogenomic analyses, J.Y.C., B.J.K. and D.Y.S.; writing—original draft preparation, J.Y.C. and C.Y.H.; manuscript editing, B.C.C. and C.Y.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2021R1F1A1059526) and by the "Development of Advanced Science and Technology for Marine Environmental Impact Assessment" of the Korea Institute of Marine Science and Technology Promotion (KIMST) funded by the Ministry of Oceans and Fisheries (202104272), Korea.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the complete genome sequence of strain HL-RS19^T are MZ820004 and CP113088, respectively. Strain HL-RS19^T was deposited in the Korean Culture Center of Microorganisms (KCCM) and Japan Collection of Microorganisms (JCM) under the number KCCM 90497^T and JCM 35710^T, respectively.

Conflicts of Interest: The authors declare no conflict of interest.

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