



Physiological, chemotaxonomic and genomic characterization of two novel piezotolerant bacteria of the family *Marinifilaceae* isolated from sulfidic waters of the Black Sea

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ARTICLE INFO

Article history:

Received 4 February 2020

Received in revised form 3 July 2020

Accepted 6 July 2020

Keywords:

Marinifilaceae

Ancylomarina euxinus sp. nov.

Labilibaculum euxinus sp. nov.

Black Sea

ABSTRACT

Diversity analyses of microbial enrichments obtained from deep sulfidic water (2000 m) collected from the Black Sea indicated the presence of eleven novel putative lineages of bacteria affiliated to the family *Marinifilaceae* of the phylum *Bacteroidetes*. Pure cultures were obtained for four strains (i.e. M1P^T, M3P, A4^T and 44) of this family, which could be grouped into two different clades based on their 16S rRNA gene sequences. All four strains were Gram-negative, rod-shaped and facultative anaerobic bacteria. The genomes of all strains were sequenced and physiological analyses were performed. All strains utilized a wide range of carbon sources, which was supported by the presence of the pathways involved in carbon utilization encoded by their genomes. The strains were able to grow at elevated hydrostatic pressure (up to 50 MPa), which coincided with increased production of unsaturated and branched fatty acids, and a decrease in hydroxy fatty acids. Intact polar lipid analysis of all four strains showed the production of ornithine lipids, phosphatidylethanolamines and capnine lipids as major intact polar lipids (IPLs). Genes involved in hopanoid biosynthesis were also identified. However, bacteriohopanepolyols (BHPs) were not detected in the strains. Based on distinct physiological, chemotaxonomic, genotypic and phylogenetic differences compared to other members of the genera *Ancylomarina* and *Labilibaculum*, it was concluded that strains M1P^T and A4^T represented two novel species for which the names *Ancylomarina euxinus* sp. nov. and *Labilibaculum euxinus* sp. nov., respectively, are proposed.

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Introduction

The Black Sea is the largest anoxic water basin on Earth. The upper surface layer (approximately the upper 70 m) of the water column is oxygenated and characterized by high biological productivity. Below this depth, oxygen concentration quickly declines to barely detectable levels [34], while the sulfide concentration increases below the redoxcline [55] to approximately 400 μM. In the deep Black Sea water column, as oxygen is lacking, anaerobic degradation of descending organic matter derived from the upper photic zone and fermentation processes become more relevant. Several studies have previously characterized the microbial community or specific microbial groups in the Black Sea water column

[29,42,50,51,54,56,57]. However, little is known about the microorganisms involved in these processes and there are almost no reports of isolates from this system.

Therefore, the current study focused on the cultivation and physiological characterization of fermentative bacteria from sulfidic waters of the Black Sea at a depth of 2000 m. Primary enrichments were established using cellulose as a carbon source, which led to the isolation of four different fermentative bacterial strains. 16S rRNA gene sequence analysis indicated that these strains belonged to the genera *Ancylomarina* and *Labilibaculum*. Strains affiliated to the genus *Ancylomarina* (M1P^T and M3P) showed the highest 16S rRNA gene sequence similarity (98.1%) to *Ancylomarina salpaludis* SHSM-M15^T. However, the average nucleotide identity (ANI) and digital DNA-DNA hybridization (DDH) between strains M1P^T and M3P were clearly below the species cut-off (95–96% and <70%) [33,40], indicating that these strains could potentially be novel members of this genus. Strains

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affiliated to the genus *Labililabaculum* (A4^T and 44) showed the highest 16S rRNA gene sequence similarity (99.1%) to *Labililabaculum manganioreducens* 59.10-2M^T, but the average nucleotide identity (ANI) and digital DNA-DNA hybridization (DDH) between strains A4^T and 44 were below the species cut-off (95–96% and <70%) [33,40], therefore, these strains are proposed as novel members of the genus *Labililabaculum*. All four strains (M1P^T, M3P, A4^T and 44) were further characterized based on genomic, chemotaxonomic, and physiological analyses compared to their nearest phylogenetic neighbors. Based on distinct physiological, genotypic and phylogenetic characteristics, we propose the description of two novel species belonging to genera *Ancylomarina* and *Labililabaculum*, namely, *Ancylomarina euxinus* sp. nov. and *Labililabaculum euxinus* sp. nov., respectively.

Materials and methods

Black Sea water sampling

A sampling campaign with the R/V Pelagia was conducted in order to collect deep waters of the Black Sea during the 64PE418 cruise (BS2017) from 27 March to 5 April 2017. A Sea-Bird SBE911C conductivity-temperature-depth (CTD) system equipped with a 24 × 12 L Niskin bottle rosette was used to collect water samples at a depth of 2000 m from station 1 (42° 53.78' N and 30° 40.72' E). Water was immediately transferred into a 20 L carboy, which was pressurized with N₂, covered with aluminum foil to protect it from the light, and stored at 10 °C (*in situ* temperature) until August 2017 when the sample was used to start enrichment cultures. Water was also kept at *in situ* temperature in N₂-overpressurized bottles (*ca.* 1.5–2.0 bar). Both the water collected at 2000 m and that kept at *in situ* temperature N₂-overpressurized was filtered (2 L) through a 0.2 µm SterivexTM filter for DNA analysis. Black Sea water samples (approximately 6 L) were also collected from the nearby station 2 (N 42° 53.8', E 30° 40.7'; 2107 m water depth) in a vertical profile with a depth range of 5 to 2000 m (22 samples in total) by using 0.2 µm SterivexTM filters (Millipore, The Netherlands).

Enrichments, isolation and 16S rRNA gene sequencing

Enrichments were carried out in 60 mL serum vials containing 20 mL of growth medium that were sealed with butyl rubber stoppers and aluminum caps. The growth medium (pH 7.0) contained cellulose (0.2 g L⁻¹), tryptone (0.2 g L⁻¹), yeast extract (0.1 g L⁻¹), CaCl₂·2H₂O (0.1 g L⁻¹), NaCl (20.0 g L⁻¹), MgCl₂·6H₂O (0.36 g L⁻¹), MgSO₄·7H₂O (0.43 g L⁻¹), KCl (0.05 g L⁻¹) and Na₂S·9H₂O (100 mg L⁻¹). Approximately 10 mL of the sampled water from the serum bottles were added to serum vials inside an anaerobic glove box (Coy Laboratory, USA). These anaerobic enrichments were kept at 10 and 20 °C in order to enrich bacterial members. After 10 days incubation, the enrichments were streaked on the agar medium and grown in anaerobic jars supplemented with Anaerocult[®] (VWR, The Netherlands) under anoxic conditions. Four bacterial strains (M1P^T, M3P, A4^T and 44) were purified by repeated streaking (5–6 times) on the agar medium.

Freshly grown pure cultures of all four strains were used for 16S rRNA gene amplification by using universal primers, as described previously [49]. The reaction mixture was analyzed by using a 3730 Genetic analyzer (Applied Biosystems) at Base-Clear, The Netherlands. Identification of the closest relatives and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EZBioCloud server [63]. Sequence alignments and phylogenetic analyses were performed as described earlier [49]. The EMBL/NCBI/DBJ accession numbers for the 16S rRNA

gene sequences of strains M1P^T, M3P, A4^T and 44 are LS999438, LS999410, LS999417, LS999409, respectively.

S rRNA gene amplicon sequencing and quantification in the Black Sea water column

To analyze the total genetic diversity of the bacterial members affiliated with the family *Marinifilaceae*, DNA was extracted *in situ* from the Black Sea water column, from the water stored at 10 °C used for the enrichments, and from the enrichment cultures themselves, and further amplified for 16S rRNA gene amplicon sequencing. The obtained 0.2 µm SterivexTM filters were extracted with a DNeasy Power Soil Kit (Qiagen, USA). For the environmental samples and the water stored in the laboratory for the enrichments, a fragment of the 16S rRNA gene was amplified by using a universal primer, as described previously [53]. Data was analyzed by the Cascabel pipeline [6], including quality assessment by FastQC [4], assembly of the paired-end reads with Pear [64], library demultiplexing, OTU clustering and representative sequence selection ('longest' method) by diverse Qiime scripts [14]. The OTU clustering algorithm was uclust [19] with an identity threshold of 97% and taxonomy was assign with BLAST [2] by using the Silva 128 release as the reference database (<https://www.arb-silva.de/>) [38]. Quantitative PCR (qPCR) was performed using the same primers as for the 16S rRNA gene amplicon sequencing analysis [53]. To extract DNA from the enrichments, 5 mL aliquots were centrifuged at 15,000 g for 10 min, the supernatant was removed and the pellet was re-suspended in 200 µL sterile Milli-Q water. In both cases, DNA was extracted using a DNeasy Power Soil Kit (Qiagen, USA) according to the manufacturer's instructions. Reads attributed to *Marinifilaceae* in the environmental samples and in the water sample kept in the laboratory to start enrichments were extracted by using Qiime [14] and, together with the 16S rRNA gene sequence of the isolated strain, were used to construct a phylogenetic tree with MEGA7, as described previously [49]. Quantitative PCR was performed on DNA extracted from the vertical profile of the water column with the same 16S rRNA gene amplicon sequencing primers used for the diversity determination, as described earlier [53]. 16S rRNA gene amplicon sequencing data of the Black Sea water columns were deposited in the NCBI under Bioproject numbers PRJNA596220 and PRJNA602617.

Genome sequencing, assembly and annotations

Genomic DNA from the pure cultures of strains M1P^T, M3P, A4^T and 44 was isolated by the method of Marmur [32]. Sequencing of the genome of all four strains was performed at the CGEB-IMR facilities (Dalhousie University, Canada), using an Illumina MiSeq (Illumina Inc) sequencing platform. Assembly of the raw sequencing data was performed on the PATRIC (Pathosystems Resource Integration Center; <https://www.patricbrc.org/>) server [59]. Genome completeness was examined by CheckM [36]. Annotation of the assembled data was performed using the Rapid Annotation using the Subsystem Technology (RAST) server (<http://rast.nmpdr.org/rast.cgi>) and PATRIC (Pathosystems Resource Integration Center; <https://www.patricbrc.org/>) [59]. The NCBI accession numbers of strains M1P^T, M3P, A4^T and 44 are QQWG00000000, WOTV00000000, WTCR00000000 and QTZN00000000, respectively.

Morphological, physiological and biochemical analyses

Morphological properties and other physiological tests, such as growth at different temperatures, pH and NaCl concentrations, were performed as described earlier [45]. To examine the growth at high hydrostatic pressure, bacterial strains were grown under a

hydrostatic pressure range of 0.1–60 MPa. Cultivation at elevated hydrostatic pressure was performed by using a “high-pressure cultivation assembly” device designed at the Royal Netherlands Institute for Sea Research, The Netherlands. The high-pressure cultivation assembly was equipped with a pressure gauge to monitor the pressure inside the pressure vessels. The pressure vessel was completely filled with liquid medium (35 mL) and inoculations were carried out under strict anaerobic conditions inside an anaerobic glove bag (Coy Lab Products, USA). The pressure vessels inoculated with cultures were set at specific hydrostatic pressures by means of a hand operated high-pressure generator using water as the hydraulic fluid. For subsampling, the vessel was carefully depressurized inside an anaerobic glove bag. Growth on organic substrates (cellulose, chitin, starch, cellobiose, sucrose, D-glucose, glycerol, pyruvate, acetate, L-glutamate, L-aspartic acid and L-lysine) was tested as described previously [45]. Vitamin (DL-pantothenate, thiamine, B₁₂ and biotin) requirements were tested by replacing the yeast extract with single and combined vitamins as growth factors. Various biochemical tests (starch/casein/chitin, oxidase, and catalase activity) were carried out as described previously [45]. Other enzymatic activities and biochemical tests were determined with an API 20A kit (bioMérieux, France) according to the manufacturer's instructions. All four bacterial strains (M1P^T, M3P, A4^T and 44) affiliated to the family *Marinifilaceae* were characterized based on a polyphasic taxonomic approach. The nearest phylogenetic neighbors of these strains (i.e. *Ancylomarina subtilis* KCTC 42257^T, “*Ancylomarina psychrotolerans*” KCTC 15504^T, *Ancylomarina salipaludis* KACC 19862^T and *Labililabaculum manganiireducens* DSM 102944^T) were obtained from the culture collections for comparative analysis in our laboratory. Pure cultures of all four strains were preserved in glycerol (20%).

Fatty acids and intact polar lipid (IPL) analyses

Cellular fatty acids of M1P^T, M3P, A4^T, 44, “*Ancylomarina psychrotolerans*” KCTC 15504^T, *Ancylomarina salipaludis* KACC 19862^T and *Labililabaculum manganiireducens* DSM 102944^T were analyzed from the cultures grown in liquid medium with pyruvate as a single carbon source. Cells were harvested by centrifugation (5000 g for 10 min at 20 °C) during the late exponential growth phase. Hydrolysis and derivatization in order to obtain fatty acid methyl esters (FAMES) were carried out as described previously [7]. FAME identification and quantification were performed using a Thermo Finnigan Trace Ultra gas chromatograph connected to a Thermo Finnigan DSQ MS as described in [9]. The determination of double-bond positions of FAMES was performed as described previously [17].

IPLs were extracted from freeze-dried biomass using a modified Bligh-Dyer procedure and were analyzed by high performance liquid chromatography-ion trap mass spectrometry (HPLC-ITMS) with additional analysis carried out to establish accurate IPL masses using an ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS), as described previously [9]. However, it should be noted that different IPLs show different MS response behaviors, and hence their relative abundance based on peak area does not necessarily reflect their absolute abundance [9].

For the detection of the presence of biohopanoids, freeze-dried biomass was directly treated with periodic acid/sodium borohydride in order to convert bacteriohopanepolyols (BHPs) into gas chromatography (GC)-amenable hopanoid alcohols (hopanols) following procedure 2 described by [41] with some modifications, and subsequently analyzed by GC and GC-MS, as described by [8]. Respiratory quinone analysis was performed as described previously [49].

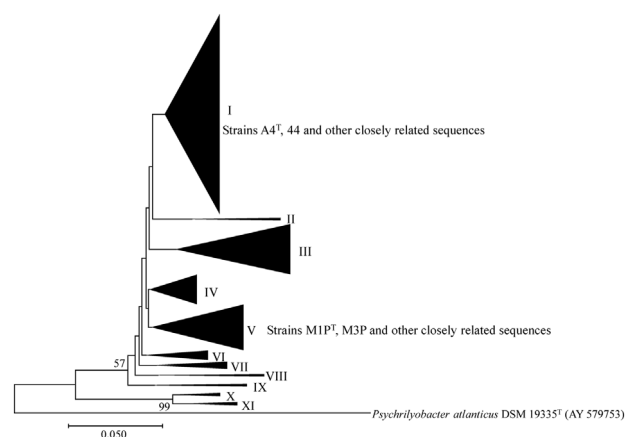


Fig. 1. Phylogenetic tree based on partial (~304 bp) 16S rRNA gene sequences obtained from Black Sea water (2000 m) and enrichment samples showing 11 lineages within the family *Marinifilaceae* and their relationship with the four isolated strains M1P^T, M3P, A4^T and 44. The tree was constructed by the neighbor-joining method using the MEGA7 software and rooted by using *Psychrobacter atlanticus* DSM 19335^T (AY 579753) as the outgroup. Numbers at nodes represent bootstrap values (based on 1000 resamplings). The length of the bar indicates 5 nucleotide substitutions per 100 nucleotides.

Results and discussion

Distribution of Marinifilaceae in the Black Sea water column

16S rRNA gene sequences affiliated to the family *Marinifilaceae* were <1% of the total microbial community in the Black Sea water column between depths of 5 to 2000 m. *Marinifilaceae* abundance (in cells per liter) in the Black Sea water column was estimated using the total concentration of 16S rRNA gene copies and the fractional abundance of *Marinifilaceae* in the set of 16S rRNA gene reads from each depth, assuming one copy of the 16S rRNA gene in the genome of this family. Maximum cell abundances were detected in the upper part of the water column (5 and 30 m) and in the upper sulfidic waters underneath the redoxcline (i.e. 130–250 m) (Supplementary Fig. S1A, S1B). However, cell abundance of *Marinifilaceae* members decreased with increasing sulfide concentration in the Black Sea at a depth of 2000 m, where oxygen was not detectable (Supplementary Fig. S1C). Phylogenetic analysis of representative sequences (>10 OTUs from each depth) affiliated to the family *Marinifilaceae* indicated that they potentially represented eleven novel putative lineages of bacteria (Fig. 1), which were closely affiliated to the family *Marinifilaceae*, with the potential to represent several novel taxa. These results indicated that Black Sea waters harbored diverse members of the family *Marinifilaceae*, which could be potentially aerobic, microaerophilic and anaerobic bacteria distributed in oxic, sub-oxic and sulfidic waters, respectively. In agreement with these findings, earlier studies also reported that members of the family *Marinifilaceae* were diverse, and included aerobic, microaerophilic and anaerobic bacteria [24,35,52,61].

Geno- and phenotypic characterization of strains isolated from the Black Sea

Although the *Marinifilaceae* members represented <1% of the prokaryotic community in the Black Sea water collected from a depth of 2000 m, the abundance of *Marinifilaceae* members increased substantially in cellobiose enrichments as the percentage of 16S rRNA gene reads attributed to this family increased to 78.7% of the total prokaryotic community. In the cultivation-based approach, four different bacterial strains (M1P^T, M3P, A4^T and 44) belonging to clades I and V (Fig. 1) were isolated. Strains M1P^T and M3P, and strains A4^T and 44, were isolated from enrich-

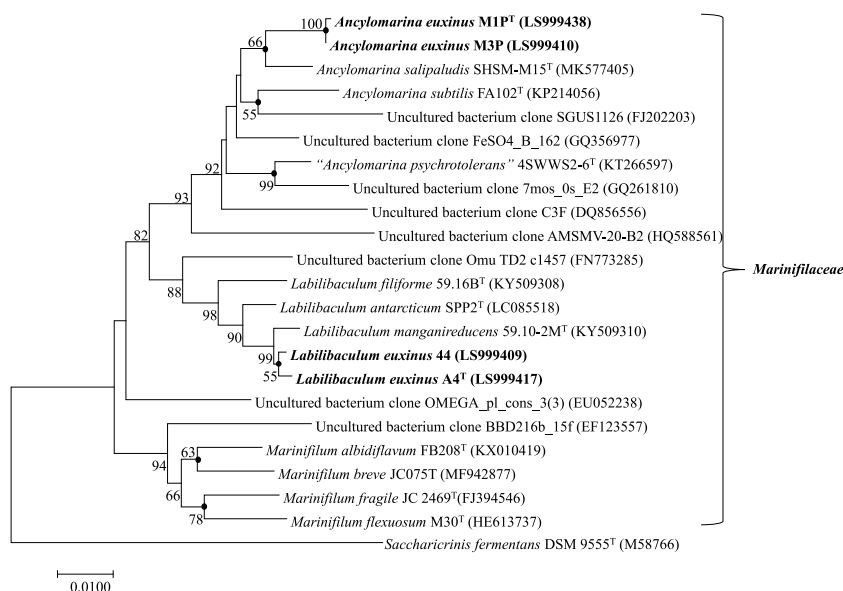


Fig. 2. Phylogenetic tree based on almost complete (>1400 bp) 16S rRNA gene sequences showing the relationship of strains M1P^T, M3P, A4^T and 44 (in bold) with strains of all formally described members of the family *Marinifilaceae* and the closest related sequences of environmental sequences available in GenBank. The tree was constructed by the neighbor-joining method using the MEGA7 software and rooted by using the *Saccharicrinis fermentans* DSM 9555^T (M58766) 16S rRNA gene sequence as an outgroup. Numbers at nodes represent the bootstrap values based on 1000 resamplings. The GenBank accession numbers for the 16S rRNA gene sequences are shown between parentheses. The length of the bar indicates 1 nucleotide substitution per 100 nucleotides. Dark circles at each node show similar grouping seen in other tree constructing methods (maximum likelihood and maximum parsimony).

ments incubated at 10 and 20 °C, respectively. All strains were purified by repeated streaking on agar medium under strict anaerobic conditions. The 16S rRNA gene phylogeny of these strains revealed that they were all members of the family *Marinifilaceae* in the order *Marinilabiliales*, class *Bacteroidia* of the phylum *Bacteroidetes* (Fig. 2). Based on BLAST search analysis of the 16S rRNA gene sequences, strains M1P^T and M3P showed 98.1% similarity to *Ancylomarina salipaludis* SHSM-M15^T, while strains A4^T and 44 showed 99.1% similarity to *Labilibaculum manganireducens* 59.10-2M^T. This would suggest that they did not represent new species. However, the draft genomes (Supplementary Table S1) of strains M1P^T and M3P (ca. 4.4 Mbp in size) and strains A4^T and 44 (ca. 5.1 Mbp) with an estimated completeness of 99–100% provided a different view, since both the average nucleotide identity (ANI) and digital DNA–DNA hybridization between strains M1P^T, M3P and *A. salipaludis* SHSM-M15^T, and strains A4^T, 44 and *L. manganireducens* 59.10-2M^T were clearly below the species cut-off (95–96% and <70%; Supplementary Tables S2 and S3) [33,40]. This indicated that these strains represented novel species of the genera *Ancylomarina* and *Labilibaculum*.

Therefore, all four strains were further characterized based on a polyphasic taxonomic analysis. The G + C content of the genomic DNA of strains M1P^T and M3P was 35.7 and 35.8%, respectively (Supplementary Table S1). A total of 22 CRISPR repeats (genomic evidence of lysogenic bacteriophage) were detected in strains M1P^T and M3P, whereas six CRISPR repeats were identified in strains A4^T and 44. The G + C content of the *Labilibaculum* strains (A4^T and 44) was 36.2%. A total of 116 genes involved in carbohydrate metabolism were identified in strains M1P^T and M3P based on the KEGG database, whereas for strains A4^T and 44 this number was 176 and 188, respectively. The distribution of genes involved in sulfur metabolism, phosphorus metabolism, potassium metabolism, lipid metabolism, DNA, RNA and protein metabolism in strains M1P^T, M3P, A4^T and 44 is listed in Supplementary Table S1. All genomes harbor a P_{II} nitrogen response system [5] and encode an amtB ammonium transporter, which is involved in the utilization of ammonia as a nitrogen source in amino acid synthesis [65]. 3-mercaptopyruvate sulfurtransferase, which may be

involved in sulfur and thiocyanate reactions [58], is also encoded in the genomes. Draft genomes of all four strains contained the genes encoding the isoprenoid 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway and the mevalonate pathway (Supplementary Fig. S2). Isoprenoid biosynthesis is essential for cell survival and the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway is considered to be the main pathway of isoprenoid biosynthesis in bacteria [28], while the mevalonate pathway is the main pathway of isoprenoid biosynthesis in eukaryotes and archaea [12,27]. The presence of both isoprenoid biosynthesis pathways in a single bacterium is unusual [30], and previous studies have indicated that this phenomenon could be associated with the production of novel isoprenoids [30]. Recent genomic studies have revealed that the mevalonate pathway is present in different bacterial groups [11,12,16,22,27,28,30,36,48] and this has been explained by horizontal gene transfer (HGT) from eukaryotic or archaeal donors [22]. However, recent phylogenomic-based studies support that this pathway was also ancestral, not only in archaea and eukaryotes but also in bacteria [22]. Hence, this peculiarity of all four strains is also interesting from an evolutionary perspective.

Strains M1P^T and M3P formed circular, smooth, pink colonies on agar medium grown under strict anaerobic conditions. Cells of these strains were 0.2–0.3 µm wide and 4.0–7.0 µm in length (Table 1). Optimum growth of strains M1P^T and M3P occurred at 18–22 °C with a growth range of 4–30 °C. Both strains required at least 0.5% NaCl for growth and could tolerate up to 5.5%. The strains differed from their closest phylogenetic affiliates (i.e. *A. salipaludis* KACC 19862^T, “*A. psychrotolerans*” KCTC^T 15504^T and *A. subtilis* KCTC 42257^T) by a relatively higher level of NaCl tolerance in the range of 0.5–5.5% (Table 1). Both strains (M1P^T and M3P) possessed gliding motility. Strains M1P^T, M3P, *A. salipaludis* KACC 19862^T and *A. subtilis* KCTC 42257^T were negative for catalase and oxidase activity, whereas “*A. psychrotolerans*” KCTC^T 15504^T was positive for catalase and oxidase activities.

Strain A4^T formed circular, smooth, light pink colonies on agar medium, whereas strain 44 formed glistening red colonies on agar medium under strict anaerobic conditions. Cells of these strains

Table 1Differentiating characteristics between strains M1P^T, M3P, *A. salipaludis* KACC 19862^T, "*A. psychrotolerans*" KCTC 15504^T and *A. subtilis* KCTC 42257^T.

Characteristics	1	2	3	4	5
Natural habitat	Deep marine water	Deep marine water	Marine sediments	Marine sediments	Salt marsh
Cell form	Rod	Rod	Rod or filamentous	Rod	Filamentous
Cell size (μm; diameter)	0.2–0.3 × 5–6.0	0.2–0.3 × 4–7.0	0.2–0.4 × 4.0–10.0	0.4–0.7 × 2.0–5.0	0.3–0.4 × 3.0–30
Optimum pH range	7.0–8.0	7.0–8.0	7.0–8.0	5.5–8.5	7.0–8.0
Optimum (range) NaCl (%)	2–3 (0.5–5.5)	2–3 (0.5–5.5)	1–2 (0.5–3.0)	1–3 (1–5.0)	1–2 (0.5–5.0)
Optimum (range) temperature (°C)	18–23 (4–30)	18–23 (4–30)	25 (10–30)	16 (4–37)	28–30 (8–33)
Gliding motility	+	+	–	–	+
H ₂ S production	–	–	–	–	+
Catalase	–	–	+	–	–
Oxidase	–	–	+	–	–
Oxygen relationship	FA	FA	FA	OA	FA
Organic substrate utilized for growth					
Starch	+	+	+	+	–
Cellobiose	+	+	+	–	–
API 20A					
Indole	–	–	+	–	+
Urease	+	+	–	+	–
D-xylose	–	–	+	+	+
Gelatin	–	–	–	–	–
Esculin	+	+	–	–	–
D-cellobiose	+	+	–	+	–
D-raffinose	–	–	–	+	–
D-sorbitol	+	+	–	+	+
D-melezitose	–	–	–	+	–
L-rhamnose	–	–	–	+	+
D-trehalose	–	–	–	+	+
G+C (mol%)	35.7	35.8	36.6	37.6	36.5

1, strain M1P^T; 2, strain M3P; 3, *Ancylomarina salipaludis* KACC 19862^T; 4, *Ancylomarina subtilis* KCTC 42257^T and 5, "*Ancylomarina psychrotolerans*" KCTC 15504^T; negative for chitinase activity; positive for glucose fermentation; utilize D-glucose, L-arabinose, mannose, lactose, saccharose, D-maltose, D-mannose; OA, obligate anaerobe; FA, facultative anaerobe; +, positive/required; –, negative/not required.

Table 2Differentiating characteristics between strains A4^T, 44, *L. manganireducens* DSM 102944^T and *L. filiforme* 59.16B^T.

Characteristics	1	2	3	*4
Natural habitat	Deep marine water	Deep marine water	Marine sediments	Marine sediments
Cell size (μm; diameter)	0.2–0.3 × 3.0–5.0	0.2–0.3 × 3.0–6.0	0.6–0.8 × 1.0–4.0	0.5–0.8 × 2.0–3.0
Optimum pH range	7.0–8.0 (6.5–9.0)	7.0–8.0 (6.5–9.0)	7.5–8.0 (5.9–8.5)	7.0–7.5 (6.0–8.5)
Optimum (range) NaCl (%)	2–3 (0.5–5.5)	2–3 (0.5–5.5)	2–3 (0.5–6.5)	0.5–1.0 (0.05–6.5)
Optimum (range) temperature (°C)	25–28 (4–35)	20–28 (4–35)	25 (4–30)	20 (4–25)
Gliding motility	–	+	–	–
Oxygen relationship	FA	FA	FA	OA
Organic substrate utilized for growth				
Cellulose	+	+	–	NA
Starch	+	+	–	+
Cellobiose	+	+	+	–
API 20A				
Indole	–	–	+	–
Urease	+	+	–	+
D-xylose	–	–	–	+
Gelatin	–	–	+	–
Esculin	+	+	+	–
D-cellobiose	+	+	+	+
D-raffinose	–	–	–	+
D-sorbitol	+	+	–	+
D-melezitose	–	–	–	+
L-rhamnose	–	–	+	+
D-trehalose	–	–	–	+
G+C (mol%)	36.2	36.1	36.7	35.8

1, Strain A4^T; 2, strain 44; 3, *Labililaculum manganireducens* DSM 102944^T; 4, *Labililaculum filiforme* 59.16B^T; negative for chitinase activity; positive for glucose fermentation; utilize D-glucose, L-arabinose, mannose, lactose, saccharose, D-maltose, D-mannose; OA, obligate anaerobe; FA, facultative anaerobe; NA, not available; +, positive/required; –, negative/not required. *Data taken from [52].

were 0.2–0.3 μm wide and 3.0–6.0 μm in length (Table 2). Growth of strains A4^T and 44 occurred from 4 °C to 35 °C, whereas no growth was detected below 4 °C or above 35 °C. The strains were negative for catalase and oxidase activity.

Growth of all strains occurred from pH 6.5–8.8 and their optimum pH range was between 7.0–8.0 (Table 1). Furthermore, growth of all strains was dependent on yeast extract, while additional vitamins were not required for growth. Sodium thiogly-

colate (0.1 g L⁻¹), sodium sulfide (100 mg L⁻¹), sodium dithionite (100 mg L⁻¹) and L-cysteine hydrochloride (100 mg L⁻¹) supported the growth of all strains as reducing agents. However, all four strains were also able to grow without reducing agents. All strains grew well when ammonium chloride (0.05%) was added as the nitrogen source in the growth media together with yeast extract.

All the tested strains grew well under strict anaerobic conditions, however, growth under microaerophilic conditions was also

observed, whereas growth in aerobic conditions was not detected. These observations suggested that they tolerated oxygen and/or grew by aerobic respiration only under microaerophilic conditions. The draft genome sequence of all four strains indicated the presence of genes encoding superoxide dismutase and catalase, which are involved in the detoxification of oxygen [13,44]. The gene encoding superoxide reductase has previously also been found in other anaerobic microorganisms [39,44]. Moreover, *cbb3*-type cytochrome c oxidase, cytochrome c oxidase subunit CcoP (EC 1.9.3.1) and cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-) were also identified and have previously been associated with microaerophilic growth in bacteria [44]. All strains were obligate chemoorganoheterotrophs. Chemolithotrophic, chemoautotrophic or phototrophic growth were not observed in any of the strains.

Reduction of sulfate to sulfide and nitrate to nitrite was not observed in the presence of glucose. All four strains (M1P^T, M3P, A4^T and 44) were also unable to reduce Fe (III) or manganese (IV). However, these strains fermented a wide range of different carbon sources (starch, cellobiose, glucose, fructose, galactose, arabinose, xylose, maltose, pyruvate, lactate and glycerol) for growth. The major end products of glucose fermentation were H₂ and CO₂. Genome analysis confirmed the presence of genes encoding enzymes responsible for the production of acetate (acetaldehyde dehydrogenase; EC 1.2.1.10), ethanol (alcohol dehydrogenase; EC 1.1.1.1), butyrate (butyrate-acetoacetate CoA-transferase subunit A; EC 2.8.3.9/ butyrate-acetoacetate CoA-transferase subunit B; EC 2.8.3.9), hydrogen and carbon dioxide (Supplementary Fig. S3) from glucose fermentation. This analysis also confirmed the presence of other genes encoding enzymes involved in hydrolysis of starch, glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle, supporting the capability of these strains to ferment a wide range of carbohydrates (Tables 1 and 2). Autotrophic growth was not possible in the tested strains with H₂/CO₂ as an electron donor and carbon source, respectively. Pyruvate was utilized as a preferred carbon source by all four strains, which was further supported by the presence of the gene encoding the pyruvate-formate lyase (EC 2.3.1.54) and the pyruvate-ferredoxin oxidoreductase (alpha, beta, delta and gamma subunits) coding genes in the genomes of the four strains. Similar to our findings, earlier studies also reported that members of the family *Marinifilaceae* were actively involved in the hydrolysis of mono-, di- and polysaccharides in different marine habitats [21,24,35,52,61]. Therefore, it appeared that these strains were involved in carbon cycling in the Black Sea together with other heterotrophic microbial communities [47,50,51].

Chemotaxonomic analyses

Strains M1P^T and M3P both contained *iso*-C_{15:1}ω8*cis*, *iso*-C_{15:0}, *iso*-C_{17:1}ω8*cis* and *iso*-β-OH C_{15:0} as predominant (*i.e.* >5%) fatty acids. Strain M1P^T also contained *iso*-β-OH C_{17:0}. They also both contained minor (<5% and >1%) amounts of C_{14:0}, *anteiso*-C_{15:0}, C_{15:0}, C_{16:1}ω7*cis*, C_{16:0}, *iso*-C_{17:1}ω6*cis*, *iso*-C_{17:1}ω8*trans* and *iso*-C_{17:0} (Table 3). Additionally, strain M1P^T contained minor amounts of β-OH C_{16:0}, while strain M3P contained minor amounts of *iso*-β-OH C_{15:1}ω8*cis*, *iso*-β-OH C_{17:0} and a lipid identified as glycine *iso*-β-OH C_{17:0} amide. The presence of *iso*-C_{17:1}ω8*cis* as a predominant fatty acid in strains M1P^T and M3P distinguished them from their nearest phylogenetic neighbors (*A. salipaludis* KACC 19862^T, *A. psychrotolerans* KCTC 15504^T, *A. subtilis* KCTC 42257^T; Table 3). Strains A4^T and 44 contained *iso*-C_{15:0}, *anteiso*-C_{15:0}, *iso*-β-OH C_{15:0} and *iso*-β-OH C_{17:0} as predominant fatty acids (>5%) with minor (<5% and >1%) amounts of *iso*-C_{15:1}ω8*cis*, C_{15:0}, C_{16:0}, *iso*-C_{17:1}ω8*cis*, β-OH C_{16:0} and *anteiso*-β-OH C_{17:0} (Table 3). Their fatty acid distributions were highly similar to that of *L. manganireducens* DSM 102944^T but differed considerably from that of *L. filiforme*

DSM 101180^T, which additionally contained C_{15:1}ω6*cis*, C_{15:1}ω8*cis*, C_{17:1}ω6*cis*, C_{17:1}ω8C, C_{18:1}ω9*cis* and C_{18:0} (Table 3).

Ornithine lipids (OLs), phosphatidylethanolamines (PEs) and capnine lipids (CpL, *i.e.* sulfur containing lipids) were present as major intact polar lipids (IPLs) in all four strains (Supplementary Table S4), while flavolipins (FL), glycine lipids (GlyL - also known as cytolipins) and phosphatidylcholines (PCs) were present in minor amounts (see Supplementary Fig. S4 for structures). The abundance of ornithine lipids and other non-phosphorous containing IPLs may be an adaptation of these strains to the strongly varying phosphorus concentrations of the Black Sea. At 2000 m (*i.e.* the depth from which the strains were isolated) the phosphorous concentration is relatively high (*i.e.* 6 μM; [10]). However, in the sub-oxic zone, where 16S rRNA gene amplicon analysis revealed that *Marinifilaceae* members were also present and occurred even more abundantly, the phosphorous concentration is low (~0.02 μM) [10] and perhaps limiting.

The presence of bacteriohopanepolyols (BHPs) was tested in strains M1P^T and A4^T by the formation of hopanols through Rohmer degradation [41] but this test was negative. This was somewhat surprising since the biosynthetic genes involved in the formation of hopanoids (see Supplementary Fig. S2) were detected in the draft genome sequence of strains M1P^T, M3P, A4^T and 44, although squalene hopene cyclase was not detected in strains M3P and 44. The analyses suggested that some of these strains may produce hopanoids under conditions other than those tested here. All the tested strains in this study contained MK-7 as the major menaquinone.

Adaptation of *Ancylomarina* sp. strain M1P^T and *Labilibaculum* sp. strain A4^T to elevated hydrostatic pressure

As all four strains were isolated from depth (*i.e.* 2000 m) in the Black Sea, where the hydrostatic pressure is approximately ~20 MPa, their ability to grow at different hydrostatic pressures was tested and, as a result, all strains grew at up to 50 MPa. Elongation in the cell size of these strains was observed when they were grown at elevated hydrostatic pressure. Such alterations in the cells might be possible due to inhibition of the polymerization of the FtsZ proteins involved in cell division at elevated hydrostatic pressure [23]. The maximum cell size of strains A4^T and 44 was measured at up to 60 μm in length at 50 MPa, whereas the cell size of strains M1P^T and M3P ranged from 20–25 μm in length at 50 MPa. One of the strategies for microbes to cope with elevated hydrostatic pressures is to change the overall lipid composition of their membranes by adjusting the degree of unsaturation, the chain length and the branching of the fatty acid membrane lipids [1,18,20,31,62]. In order to evaluate the effect of hydrostatic pressure on the lipid membrane of these strains, the fatty acid and IPL composition of *Ancylomarina* sp. strain M1P^T and *Labilibaculum* sp. strain A4^T were analyzed at atmospheric pressure (0.1 MPa) and at the *in situ* hydrostatic pressure (*i.e.* 20 MPa). Between 0.1 MPa and 20 MPa, the relative abundance of unsaturated fatty acids in strain M1P^T increased from 27.7 to 39.5% (Supplementary Table S5). For strain A4^T, a smaller increase in unsaturated fatty acids of between 6.7 to 9.5% with increasing pressure was observed (Supplementary Table S5). Previous studies have reported a similar relationship between growth at elevated hydrostatic pressure and an increase in the proportion of unsaturated fatty acid [18,62], which is thought to be related to maintenance of membrane fluidity and permeability in order to supply appropriate solutes under such conditions [18]. Additionally, a decrease in the relative abundance of hydroxy fatty acids with increasing pressure was observed in both strains M1P^T and A4^T, from 31.0 to 13.6% and 33.6 to 18.5%, respectively (Supplementary Table S5). This adaption is also in agreement with previous

Table 3

Cellular fatty acid compositions (%) of strains M1P^T, M3P, *A. salipaludis* KACC 19862^T, "*A. psychrotolerans*" KCTC 15504^T, *A. subtilis* KCTC 42257^T, A4^T, 44, *Labililabaculum manganireducens* DSM 102944^T and *Labililabaculum filiforme* DSM 101180^T.

Fatty acid	Relative abundance								
	1	2	3	4	5	6	7	8	9
<i>iso</i> -C _{13:0}	0.5	0.7	0.3	0.1	3.9	0.4	1.0	0.8	–
<i>anteiso</i> -C _{13:0}	–	–	0.1	1.4	–	0.1	0.2	0.2	–
<i>iso</i> -C _{14:0}	–	–	5.4	0.4	0.6	–	0.1	0.3	–
C _{14:0}	1.0	1.0	3.2	0.4	–	0.1	0.3	0.3	0.4
<i>iso</i> -C _{15:1} ω8 <i>cis</i>	8.6	11.9	–	8.7	6.0	3.5	4.0	3.4	12.4
<i>anteiso</i> -C _{15:1} ω8 <i>cis</i>	0.5	0.4	–	2.7	–	0.5	0.3	0.4	4.4
<i>iso</i> -C _{15:0}	33.8	33.8	41.3	25.3	46.7	45.5	45.0	45.8	23.2
<i>anteiso</i> -C _{15:0}	1.0	1.2	19.4	33.6	6.3	7.7	6.3	10.1	14.5
C _{15:1} ω6 <i>cis</i>	–	0.1	–	0.5	–	–	–	–	5.7
C _{15:1} ω8 <i>cis</i>	–	0.1	–	0.3	–	–	–	–	3.0
C _{15:0}	1.2	1.1	0.2	2.6	–	1.7	1.5	1.8	11.0
<i>iso</i> -C _{16:1} ω11 <i>cis</i>	–	–	5.7	–	–	–	–	–	–
<i>iso</i> -C _{16:0}	–	–	2.2	0.1	TR	0.3	0.4	1.8	–
C _{16:1} ω7 <i>cis</i>	1.2	1.7	1.6	–	–	0.5	0.4	0.2	–
C _{16:1} ω11 <i>cis</i>	–	–	6.2	–	–	–	–	–	–
C _{16:0}	2.6	1.9	3.0	–	–	2.2	1.9	1.6	1.4
<i>iso</i> -C _{17:1} ω6 <i>cis</i>	1.3	1.8	–	0.1	–	0.1	0.3	0.4	–
<i>iso</i> -C _{17:1} ω8 <i>cis</i>	13.4	15.9	–	1.2	–	1.5	1.8	4.7	4.7
<i>iso</i> -C _{17:1} ω8 <i>trans</i>	1.8	2.2	–	0.7	–	0.5	0.3	–	–
<i>iso</i> -C _{17:1} ω12 <i>cis</i>	–	–	3.2	–	–	–	–	–	–
<i>iso</i> -C _{17:1} ω12 <i>trans</i>	–	–	2.5	–	–	–	–	–	–
<i>iso</i> -C _{17:0}	1.0	1.0	1.9	0.1	–	0.9	1.1	3.1	0.3
<i>anteiso</i> -C _{17:0}	0.1	0.1	2.4	0.3	–	0.2	0.2	1.1	–
C _{17:1} ω6 <i>cis</i>	0.1	0.1	–	0.1	–	–	–	–	2.5
C _{17:1} ω8 <i>cis</i>	–	–	–	–	–	–	–	–	2.1
C _{18:1} ω9 <i>cis</i>	0.1	–	0.3	0.1	–	–	0.1	–	1.9
<i>iso</i> -β-OH C _{15:1} ω8 <i>cis</i>	0.7	1.2	–	0.1	–	0.1	–	–	–
<i>iso</i> -β-OH C _{15:0}	13.5	13.1	0.2	10.7	19.9	8.7	10.7	5.7	–
<i>anteiso</i> -β-OH C _{15:0}	0.3	0.3	–	2.5	–	0.7	1.1	0.5	–
β-OH C _{15:0}	0.2	0.1	–	0.2	1.1	–	0.1	–	–
β-OH C _{16:0}	2.0	0.4	–	0.5	–	1.5	1.5	1.5	–
<i>iso</i> -β-OH C _{17:0}	12.3	2.3	–	1.2	6.5	18.3	16.1	10.7	–
<i>anteiso</i> -β-OH C _{17:0}	0.3	0.1	–	2.5	–	3.4	2.9	2.7	–
Glycine <i>iso</i> -β-OH C _{17:0} amide	–	4.6	–	–	–	0.3	0.7	–	–

1, Strain M1P^T; 2, strain M3P; 3, *A. salipaludis* KACC 19862^T; 4, "*A. psychrotolerans*" KCTC 15504^T; 5, *A. subtilis* KCTC 42257^T; data taken from [35]; 6, A4^T; 7, 44; 8, *Labililabaculum manganireducens* DSM 102944^T; 9, *Labililabaculum filiforme* DSM 101180^T; data taken from [52]; TR, traces; –, absent/not detected.

findings when marine bacteria were grown under high hydrostatic pressure [1]. The third observed change in the fatty acid distribution of both strains in response to increased hydrostatic pressure was the relative abundance of branched fatty acids. Between 0.1 and 20 MPa this increased from 90.4 to 94.9% in M1P^T and from 93.1 to 94.3% in A4^T. The average fatty acid chain length did not change with pressure (nor vary between strains) and it was consistently around 15.5. The results of this study demonstrated that changes in the degree of unsaturation, branching and hydroxylation of fatty acids all represented adaptation strategies employed by the bacteria isolated in this study for dealing with the high hydrostatic pressure in the deep waters of the Black Sea.

Strains M1P^T and A4^T contained OLs together with three other aminolipids: C₁₆ (sulfur containing lipids), FLs and GlyLs (also known as cytolipins; Supplementary Table S4). The abundant presence of OLs in particular has previously been suggested to be bacterial adaptation to phosphate limitation [15,26,43,46], as well as regulation of the bacterial membrane surface charge and hydrophobicity, ultimately reducing bacterial susceptibility to antimicrobials and enhancing bacterial biofilm formation [26]. However, to date, there have been no reports of changes in the relative abundance of OLs or other aminolipids in response to elevated hydrostatic pressure. In this study, when strain M1P^T was grown at 20 MPa, the OL abundance increased from 62.5 to 73.8%. Conversely, when strain A4^T was grown at 20 MPa, the percentage of OLs decreased from 48.8 to 41.9% (Supplementary Table S4). The C₁₆ increased in relative abundance with increasing pressure, in both M1P^T (13.3 to 16.6%) and A4^T (9.8 to 11.9%). Conversely, both the FLs and GlyLs decreased in both strains in response to ele-

vated hydrostatic pressure (20 MPa). These results suggested that the aminolipids in the cell membrane may be affected by hydrostatic pressure but, as the effect was not consistent between the two strains or between the different type of aminolipids, it was not possible to draw conclusions on the nature of this phenomena, and further studies will be required in order to assess their formation under elevated hydrostatic pressure in different species. However, the results did provide evidence that the bacterial strains isolated in this study contributed to the OLs pool previously detected in the Black Sea anoxic zone [42].

In addition to the aminolipids, phospholipids were also detected, predominantly PEs (Supplementary Table S4). The relative abundance of PEs decreased with increasing pressure from 11.8 to 4.4% in strain M1P^T. This was consistent with a previously reported membrane lipid adaptation of a piezotolerant *Pseudomonas* spp., also isolated from the deep sea [25]. In contrast, the relative abundance of PEs in strain A4^T increased with elevated hydrostatic pressure, concomitant with the decrease in OLs. Similar observations were also noted by Kaneko et al. [25] for *Pseudomonas* sp. grown at elevated temperature and elevated hydrostatic pressure. A decrease in the relative abundance of the PCs was also observed in both strains under elevated hydrostatic pressure, in contrast to an earlier finding that indicated an increase in relative abundance of PCs in response to elevated hydrostatic pressure [1].

Generally, it could be concluded that *Marinifilaceae* member strains M1P^T and A4^T described here deployed different lipid adaptation mechanisms to high hydrostatic pressure both in comparison to each other and to previously reported piezophilic proteobacte-

Table 4Descriptions of *Ancylomarina euxinus* sp. nov. and *Labilibaculum euxinus* sp. nov.

Genus name	<i>Ancylomarina</i>	<i>Labilibaculum</i>
Species name	<i>euxinus</i>	<i>euxinus</i>
Specific epithet	<i>euxinus</i>	<i>euxinus</i>
Species status	sp. nov.	sp. nov.
Species etymology	<i>euxinus</i> (eu'xi.nus. from Gr. adj. <i>euxeinos</i> , hospitable, used to describe the Black Sea [Pontos Euxeinos]; L. adj. <i>euxinus</i> , hospitable, in reference to the Black Sea).	<i>euxinus</i> (eu'xi.nus. from Gr. adj. <i>euxeinos</i> , hospitable, used to describe the Black Sea [Pontos Euxeinos]; L. adj. <i>euxinus</i> , hospitable, in reference to the Black Sea).
Description of the new taxon and diagnostic traits	Cells are motile small rods (0.2–0.3 × 4.0–7.0 µm 4.0–7.0 µm). Gram-negative, facultative anaerobic and piezotolerant bacterium. Forms pink pigmented colonies on agar medium. Negative for catalase, oxidase and chitinase activity. Indole production and denitrification are negative. Growth occurs between pH 6.5–8.8 (optimum 7.0–8.0). Tolerates up to 5.5% NaCl with optimum growth at 2–3%. Optimum growth occurs at 18–23 °C (range 4–30 °C). Chemoorganoheterotrophy is the only growth mode. Sodium sulfide, sodium dithionite and L-cysteine hydrochloride also support growth as reducing agents. Negative for H ₂ S production. D-glucose, D-lactose, D-maltose, salicin, L-arabinose, D-cellobiose, sucrose, starch, D-mannose, L-lysine, L-glutamate, L-aspartate and acetate are utilized for growth. Growth is negative with D-melezitose, D-raffinose, D-sorbitol, L-rhamnose and D-trehalose. Ammonium chloride is a good source of nitrogen for growth. Yeast extract is required for growth. Added vitamins are not required for growth. Genes for 2C-methyl-D-erythritol 4-phosphate (MEP) and the mevalonate pathway of IPP biosynthesis are present in the genome sequence. <i>iso</i> -C _{15:1} ω8Ccis, <i>iso</i> -C _{15:0} , <i>iso</i> -C _{17:1} ω8Ccis, <i>iso</i> -β-OH C _{15:0} and <i>iso</i> -β-OH C _{17:0} are present as predominant fatty acids (>5%) MK-7 is the predominant menaquinone. Ornithine lipids, phosphatidylethanolamines, capnine lipids and flavolipins are major polar lipids (>5%).	Cells are motile small rods (0.2–0.3 × 3.0–6.0 µm). Gram-negative, facultative anaerobic bacterium. Growth at elevated hydrostatic pressure (~50 MPa) has been detected. Negative for catalase, oxidase and chitinase activity but positive for cellulase and amylase activity. Indole production and denitrification are negative. Growth occurs between pH 6.5–9.0 (optimum 7.0–8.0). Tolerates up to 5.5% NaCl with optimum growth at 2–3%. Optimum growth occurs at 20–28 °C (range 4–35 °C). Chemoorganoheterotrophy is the only growth mode. Negative for H ₂ S production. Cellulose, D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylose, L-arabinose, cellobiose, sucrose, starch, D-mannose, L-glutamate, L-aspartate, L-lysine and acetate are utilized for growth. Growth is negative with D-melezitose, D-raffinose, D-sorbitol, L-rhamnose and D-trehalose. Yeast extract is required for growth. Additional vitamins are not required for growth. Genes for 2C-methyl-D-erythritol 4-phosphate (MEP) and the mevalonate pathway of IPP biosynthesis are present in the genome. <i>iso</i> -C _{15:0} , <i>anteiso</i> -C _{15:0} , <i>iso</i> -β-OH C _{15:0} and <i>iso</i> -β-OH C _{17:0} are present as predominant fatty acids (>5%). MK-7 is the predominant menaquinone. Ornithine lipids, phosphatidylethanolamines and capnine lipids are major polar lipids (>5%).
Country of origin	Bulgaria	Bulgaria
Region of origin	Black Sea	Black Sea
Date of isolation	11/01/2018	22/11/2017
Source of isolation	Sulfidic waters of the Black Sea	Sulfidic waters of the Black Sea
Sampling date	27/03/2017 to 05/04/2017	27/03/2017 to 05/04/2017
Latitude	42° 53.78' N	42° 53.78' N
Longitude	30° 40.72' E	30° 40.72' E
16S rRNA gene accession nr.	LS999438 (strain M1P ^T), LS999410 (strain M3P)	LS999417 (strain A4 ^T), LS999409 (strain 44)
Genome accession number	QQWGO00000000 (strain M1P ^T), WOTV000000000 (strain M3P)	WTCR000000000 (strain A4 ^T), QTZN000000000 (strain 44)
Genome status	Incomplete	Incomplete
Genome size	4,403,951 bp (strain M1P ^T) and 4,351,206 bp (strain M3P)	5,055,185 bp (strain A4 ^T) and 5,151,748 (strain 44)
GC mol%	35.7 (strain M1P ^T) and 35.8 (strain M1P ^T)	36.2 (strain A4 ^T) and 36.1 (strain 44)
Number of strains in study	2	2
Source of isolation of non-type strains	Sulfidic waters (Black Sea)	Sulfidic waters (Black Sea)
Information related to the Nagoya Protocol	NA	NA
Designation of the Type Strain	M1P ^T	A4 ^T
Strain Collection Numbers	M1P ^T = JCM 32614 ^T = KCTC 15718 ^T	A4 ^T = JCM 32481 ^T = KCTC 15662 ^T

rial members [1,3,18,20,25,31,37,60,62], highlighting the complex nature of membrane lipid adaption.

Conclusions

All four strains (M1P^T, M3P, A4^T and 44) isolated in this study utilized a wide range of carbon sources, which suggested that they played an important role in the carbon cycle in the sulfidic, deep waters of the Black Sea. IPL analysis confirmed the abundance of ornithine lipids and other non-phosphorous containing IPLs, which could aid in the survival of these strains under the varying phosphorus conditions in the Black Sea [10]. The demonstration of growth at elevated hydrostatic pressure confirmed that the isolated strains were adapted to the high hydrostatic pressure existing in the deep waters of the Black Sea. However, they may also excel in the sub-oxic water column. Furthermore, the presence of both isoprenoid biosynthesis pathways (*i.e.* MEP and

mevalonate) could be important with respect to their sustainable lifestyle in their natural environment. In addition to these unique properties, all strains clearly differed from their closest phylogenetic neighbours with respect to NaCl range, temperature range, pH tolerance range and fatty acid profiles, as well as organic carbon source utilization (Tables 1–3; Supplementary Tables S1–S5). Therefore, based on distinct morphological, physiological, chemotaxonomic and phylogenetic differences from their closest phylogenetic neighbours (Tables 1–2; Fig. 2; Supplementary Tables S1–S5), we propose strains M1P^T and A4^T as two novel species in the family *Marinifilaceae*, for which the names *Ancylomarina euxinus* sp. nov., and *Labilibaculum euxinus* sp. nov. are proposed, respectively. Descriptions of *Ancylomarina euxinus* sp. nov., and *Labilibaculum euxinus* sp. nov. are given in Table 4. Furthermore, we also propose the emended description of the genera *Ancylomarina* and *Labilibaculum* with additional characteristics, as given below:

Emended description of the genus *Ancylomarina* Wu et al., 2016

The description of the genus is as given by Wu et al., 2016 [60] with the following modifications. Some species are piezotolerant. Ornithine and capnine lipids are present as major intact polar lipids together with minor amounts of flavolipins, glycine lipids (also known as cytolipins) and phosphatidylcholines in some strains.

Emended description of the genus *Labilibaculum* Vandieken et al., 2018

The description of the genus is as given by Vandieken et al., 2018 [52] with the following modifications. Some species are piezotolerant. Ornithine lipid is present as the major intact polar lipid together with minor amounts of flavolipins, glycine lipids (also known as cytolipins) and phosphatidylcholines in some strains.

Funding information

This research was supported by the SIAM Gravitation Grant (024.002.002) from the Dutch Ministry of Education, Culture and Science (OCW) to JSSD and LV, and by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 694569 – MICROLIPIDS) to JSSD.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The Korean Collection for Type Cultures (KCTC) is greatly acknowledged for providing the *Ancylomarina subtilis* KCTC 42257^T and “*Ancylomarina psychrotolerans*” KCTC 15504^T strains as a gift for comparative analysis under our laboratory conditions. We thank the Korean Agricultural Culture Collection (KACC) for providing *Ancylomarina salipaludis* KACC 19862^T for comparative analysis, also under our laboratory conditions. The authors acknowledge Marianne Baas for help with the fermentation product (gas phase) analysis and to Sanne Vreugdenhil and Maartje Brouwer for their support with the molecular genetic analyses. Daan van Vliet (WUR) is acknowledged for providing the novel species name etymology. Alejandro Abdala is acknowledged for helping with the bioinformatics analyses. We would like to thank the captain, crew and scientific participants of Black Sea expedition-2017 on board the *R/V Pelagia* for sampling and technical support. Johan van Heerwaarden (NIOZ) is greatly acknowledged for devising the “high pressure cultivation” assembly.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.syapm.2020.126122>.

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