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TAXONOMIC DESCRIPTION
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Flavobacterium salilacus sp. nov., isolated from surface water of a hypersaline lake, and descriptions of *Flavobacterium salilacus* subsp. *altitudinum* subsp. nov. and *Flavobacterium salilacus* subsp. *salilacus* subsp. nov.

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Abstract

Two yellow-pigmented, Gram-stain-negative, aerobic, rod-shaped bacteria were isolated from the water of the hypersaline Chaka Salt Lake (strain SaA2.12^T) and sediment of Qinghai Lake (strain LaA7.5^T), PR China. According to the 16S rRNA phylogeny, the isolates belong to the genus *Flavobacterium*, showing the highest 16S rRNA sequence similarities to *Flavobacterium arcticum* SM1502^T(97.6–97.7%) and *Flavobacterium suzhouense* XIN-1^T(96.5–96.6%). Moreover, strains SaA2.12^T and LaA7.5^T showed 99.73% 16S rRNA sequence similarity to each other. Major fatty acids, respiratory quinones and polar lipids detected in these isolates were iso-$C_{15:0}$, menaquinone-6 and phosphatidylethanolamine, respectively. Strains SaA2.12^T and LaA7.5^T showed significant unique characteristics between them as well as between the closest phylogenetic members. The highest digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values between SaA2.12^T and its closest neighbours were 25.3 and 82.8%, respectively; whereas these values (highest) between LaA7.5^T and its closest members were 25.2 and 82.8%, respectively. The dDDH and ANI values between strains SaA2.12^T and LaA7.5^T were calculated as 75.9 and 97.2%, respectively. Therefore, based on polyphasic data, we propose that strain SaA2.12^T represents a novel species with the name *Flavobacterium salilacus* sp. nov., with the type strain SaA2.12^T (=KCTC 72220^T=MCCC 1K03618^T) and strain LaA7.5^T as a subspecies within novel *Flavobacterium salilacus* with the name *Flavobacterium salilacus* subsp. *altitudinum* subsp. nov., with the type strain LaA7.5^T (=KCTC 72806^T=MCCC 1K04372^T). These propositions automatically create *Flavobacterium salilacus* subsp. *salilacus* subsp. nov. with SaA2.12^T (=KCTC 72220^T=MCCC 1K03618^T) as the type strain.

INTRODUCTION

The genus *Flavobacterium* belonging to *Cytophaga–Flavobacterium–Bacteroides* (C/F/B) group of the phylum *Bacteroidetes* [1, 2] is the type genus of family *Flavobacteriaceae* and was first proposed by Bergey *et al.* [3]. Since then, the description of this genus has been revised several times [4–7]. Members of the genus *Flavobacterium* are generally characterized as yellow-pigmented, Gram-stain-negative, non-spore forming, straight or slightly curved rod-shaped, characteristically either non-motile or motile by gliding, unable to grow in anaerobic condition and contain menaquinone-6 (MK-6) as the only or major respiratory quinone. Members belonging to this genus contain iso-$C_{15:0}$ as one of the predominant fatty acids, phosphatidylethanolamine as the major polar lipid and the G+C contents of the genomic DNA ranged from 30 to 52 mol% [7]. Recently the genus *Flavobacterium* has expanded considerably and during the preparation of this manuscript (March 2020), this genus comprises 224 species with validly published and correct names (http://www.bacterio.net/flavobacterium.

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Keywords: *Flavobacterium*; polyphasic analysis; novel subspecies; hypersaline lake; genome sequence.
Abbreviations: ANI, average nucleotide identity; APL, unidentified aminophospholipid; dDDH, digital DNA–DNA hybridization; GL, unidentified glycolipid; L, unidentified lipid; LB, Luria–Bertani; MA, marine agar; MB, marine broth; PE, phosphatidylethanolamine; PK, polyketide; PKS, polyketide synthase; PL, unidentified phospholipid; R2A, Reasoner's 2A; SM, secondary metabolite; TSA, tryptone soya agar; TSB, tryptic soy broth.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Flavobacterium salilacus* (SaA2.12^T) is MN864570 and for *Flavobacterium salilacus* subsp. *altitudinum* (LaA7.5^T) it is MN864568. The GenBank accession numbers for the genome sequences of *Flavobacterium salilacus* (SaA2.12^T), *Flavobacterium salilacus* subsp. *altitudinum* (LaA7.5^T) and *Flavobacterium arcticum* KCTC 42668^T are SKBP00000000, JAACYB000000000 and SKBO00000000, respectively.
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Seven supplementary figures are available with the online version of this article.

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html), with *Flavobacterium aquatile* as the type species [3, 4, 8]. Members of this genus are physiologically diverse; they can be halophilic, psychrophilic, psychrotolerant or mesophilic [9]. Because of their broad physiological diversity, members of this genus have been isolated from a range of ecological niches [10]. However, no members of this genus have been isolated from hypersaline environments [1, 11]. In addition, no subspecies of the genus *Flavobacterium* has been recorded to date (www.bacterio.net/flavobacterium.html). During our investigation of bacterial diversity from inland saline aquatic environments in PR China, we isolated two presumptive *Flavobacterium* strains designated SaA2.12^T and LaA7.5^T from Chaka Salt Lake and Qinghai Lake, respectively. This present study was designed to investigate the taxonomic position of these two novel strains following polyphasic techniques and proposed them as novel members of the genus *Flavobacterium*.

STRAIN ISOLATION, CULTURE CONDITIONS AND PRESERVATION

In August 2017, strain SaA2.12^T was isolated from surface water from Chaka Salt Lake (36° 44′ 45.33″ N, 99° 5′ 4.46″ E) and LaA7.5^T from shallow water sediment of Qinghai Lake (36° 37′ 59.53″ N, 100° 8′ 58.28″ E), Qinghai Province, PR China. Chaka Salt Lake is an anathalassohaline lake situated at high elevation (3214 m above sea level [12]) and exhibits a salinity from 0.2 g l^{-1} (at the depth of 9 m) to 325 g l^{-1} (at the top) [13, 14], which makes it a hypersaline lake (salinity >35 g l^{-1}). On the other hand, Qinghai Lake, situated at an altitude of 3205 m above sea level, is a saline (1 g l^{-1}<salinity<35 g l^{-1}) and alkaline lake (9.0 <pH<12), and the salinity and pH of Qinghai Lake are recorded as 12.9 g l^{-1} and pH 9.1, respectively [13, 15]. The combination of high elevation and salinity makes these two lakes ideal sites for studying halophilic bacterial diversity. After collecting the samples, approximately 1 g sediment from Qinghai Lake was suspended in sterile artificial seawater. Subsequently, 100 µl of this suspension and 100 µl water from Chaka Salt Lake was directly inoculated and spread onto modified 2216E agar plates (0.05 g tryptone, 0.01 g $FePO_4$, 0.1 g yeast extract, 3.5 g sea salt, 2.2 g agar per litre ddH_2O; pH 6.5~7.0) and then incubated at room temperature for 3–7 days. After 7 days of incubation, numerous colonies with different colony morphology were obtained. Among these various colonies, two circular, around 2–4 mm and yellowish-colour colonies were picked and designated SaA2.12^T and LaA7.5^T. Due to weak growth on the modified 2216E agar plate, these isolates were then purified, routinely cultured on marine agar 2216 (MA; BD), and were stored at −80 °C in marine broth 2216 (MB; BD) containing 25% (v/v) glycerol for preservation and further studies. Strains SaA2.12^T and LaA7.5^T have been deposited at the Marine Culture Collection of China (MCCC) and the Korean Collection for Type Cultures (KCTC).

MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Cellular, as well as flagellar morphologies of SaA2.12^T and LaA7.5^T were examined by using light microscopy (E100, Nikon) and transmission electron microscopy (JEM1230, JEOL) with cells grown on MA for 2–3 days (log phase) at 28 °C, which has been described previously [10]. ImageJ software (National Institute of Health, version 1.6.0) was used for measuring the size of the cells. The colony morphology of these strains was observed on MA after incubation at 28 °C for 3–4 days. Gram staining was performed using a BD Gram stain kit according to the manufacturer's protocol. Growth of novel isolates SaA2.12^T and LaA7.5^T was tested on MA, tryptone soya agar (TSA; Oxoid), Reasoner's 2A (R2A) agar (Oxoid), nutrient agar (NA; Oxoid), Luria–Bertani (LB) agar (Difco) and typtone–yeast extract–salt (TYS) agar plates (containing 0.5% tryptone, 0.1% yeast extract, 1.5% agar, artificial seawater) at 28 °C for 3–7 days under aerobic conditions. Moreover, growth at different temperatures (4, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C), pH (pH 5.0–10.0, at 0.5 intervals) and salt (NaCl) concentration (0–11.0%, w/v, at 0.5 intervals) was accessed in TYS broth (containing 0.5% tryptone, 0.1% yeast extract, 0.5% $MgCl_2$, 0.2% $MgSO_4$, 0.05% $CaCl_2$, 0.1% KCl and 0.0001% $FeSO_4$) [16] at 28 °C by reading OD_{600} values over 7 days of incubation. For liquid culture, MB was not used due to the turbidity of the broth. The pH for growth was adjusted by MES (for pH 5.5–6.0), MOPS (for pH 6.5–7.5), Tricine (for pH 8.0–8.5) and Bis–Tris propane (for pH 9.0–9.5) buffers at 30 mM final concentration [10]. Motility and gliding motility was investigated by culturing cells in a motility test medium [17] and following the hanging-drop method [18], respectively. Growth under anaerobic conditions was examined with an anaerobic box (AnaeroPack-Anaero; Mitsubishi Gas Chemical Co.). Thus, strains were cultured on MA and incubated in the anaerobic box at 28 °C for 7 days according to the manufacturer's instruction. 1% (w/v) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride was used to determine the oxidase activity by observing the colour shift on filter paper; and catalase activity was examined in a 3% H_2O_2 solution by observing bubble production [19]. Flexirubin-type pigment production was determined with KOH solution (20%) and Congo red adsorption was tested with Cong-red dye (0.01%, w/v) [18]. Hydrolysis of Tween 20, Tween 40, Tween 60 and Tween 80 (1.0%, w/v) were performed according to Smibert and Krieg [20]; hydrolysis of casein (2.0% skimmed milk, w/v), tyrosine (0.5%, w/v), hypoxanthine (0.05%, w/v) and xanthine (0.05%, w/v) was investigated according to Kurup and Babcock's method [21]; hydrolysis of starch (1.0%, w/v) and reduction of nitrate to nitrite were examined following methods described by Barrow and Feltham [17]; hydrolysis of CM-cellulose (1.0%, w/v), filter paper (crystalline cellulose), methyl red and Voges–Proskauer reactions were determined after Chen *et al.* [22]. Moreover, carotenoid pigments from novel strains SaA2.12^T and LaA7.5^T were extracted, purified and analysed following the methods described previously [10]. Additional physiological, biochemical and enzyme tests of these novel

strains were also determined parallel with type strains using API ZYM, API 20NE and API 50CH (bioMérieux) kit systems according to the manufacturer's instructions.

Cells of novel isolates SaA2.12^T and LaA7.5^T were Gram-stain-negative, catalase-oxidase positive, aerobic, devoid of flagella and motile by gliding. Photomicrographs of the light microscopy and transmission electron microscopy showed that cells of these isolates were non-spore forming rods and the cells of SaA2.12^T and LaA7.5^T were 0.5–0.6 µm×1.2–1.4 µm, and 0.4–0.6 µm×0.8–1.5 µm, respectively (Fig. S1, available in the online version of this article). Growth of the strains SaA2.12^T and LaA7.5^T was spontaneous on MA, TSA, R2A agar and NA, and weak on LB agar. Surprisingly, the two novel isolates grew very well in TYS broth but on the TYS agar plate, they grow weakly. Thus, in the case of plate culture, we used MA and, in the case of liquid culture, we used TYS broth instead of MB. Though many common properties were found among the two novel isolates and the closest phylogenetic neighbours, several unique properties were also observed which differentiate these novel isolates from each other and from their closest phylogenetic neighbours (Table 1). For instance, strains SaA2.12^T could be separated from all other studied strains by lipase (C14) activity, methyl red activity, urease activity, assimilation of mannose and acid production from potassium 2-ketogluconate; strain LaA7.5^T could be distinguished from all studied strains by hydrolyses of Tween 80 and casein (Table 1). According to the spectroscopic profile (Fig. S2) and the mass spectrum (*m/z*=569.4 $[M+H]^+$) of the pigments from the two novel strains, their main carotenoid was identified as zeaxanthin, a type of carotenoid reported in several members of genus *Flavobacterium* and also in some other members of the family *Flavobacteriaceae* [10]. Detailed phenotypic characteristics that differentiate strain SaA2.12^T and LaA7.5^T from their closest neighbour are presented in Fig. S1, Table 1 and in the species description.

PHYLOGENETIC ANALYSIS

For phylogenetic analysis and 16S rRNA gene sequence amplification, the Takara genomic DNA extraction kit (Kusatsu) was used to extract the genomic DNA from strains SaA2.12^T and LaA7.5^T. From this genomic DNA, the 16S rRNA gene fragment of these strains was amplified by the PCR method using the universal bacterial primers 27F and 1492R [23]. Subsequently, the PCR products were purified with a commercial gel extraction kits (Cwbio), ligated into the pMD-19T vector (Takara) for sequencing to get the full-length 16S rRNA gene sequence. The resultant almost-complete 16S rRNA gene sequences were compiled using SeqBuilder software (DNASTAR version 7.1.0) and then compared to find their taxonomic position by the BLASTN program (www.ncbi.nlm.nih.gov/) [24] as well as the EzBiocloud server (www.ezbiocloud.net/) [25]. Afterward, 16S rRNA gene sequences of closely related and validly published *Flavobacterium* species were retrieved from the EzBioCloud GenBank database [25] and subsequently subjected to multiple sequences alignments using CLUSTAL_X 2.1 software [26]. Phylogenetic comparison of the obtained sequences was performed by the MEGA 7.0 program [27]. The evolutionary history of the 16S rRNA gene sequences of all obtained strains was investigated by following the neighbour-joining [28], maximum-likelihood [29] and maximum-parsimony [30] methods using *Chryseobacterium gleum* ATCC 35910^T (AM232812) as an outgroup and Kimura's two-parameter model was used to calculate the genetic evolutionary distance [31]. Moreover, 1000 replicates were used to estimate the bootstraps values [32].

The almost-complete 16S rRNA gene sequences of strain LaA7.5^T and SaA2.12^T contain 1477 bp (GenBank/EMBL/DDBJ accession numbers MN864568 and MN864570, respectively). The 16S rRNA gene sequences analysis by multiple sequence alignment showed that strains SaA2.12^T and LaA7.5^T shared a 99.73% (1473/1477bp) sequence similarity, a value corresponding to four nucleotides differences at 732, 1112, 1113 and 1114 positions. Preliminary comparison of the 16S rRNA gene sequences in BLASTN and the EzBioCloud server indicated the affiliation of these two novel strains with the genus *Flavobacterium*. Moreover, strain SaA2.12^T showed the highest nucleotide sequence similarities to *F. arcticum* SM1502^T (97.6%) and *F. suzhouense* XIN-1^T (96.6%), while LaA7.5^T is closely related to *F. arcticum* SM1502^T (97.7%) and *F. suzhouense* XIN-1^T (96.5%), significantly below the 98.65% cut-off value suggested for differentiating two species by Kim *et al.* [33]. The similarity between these strains and other validly described *Flavobacterium* species were below 96.1%. Analogously, all the phylogenetic trees generated revealed that these isolates formed a distinct cluster with *F. arcticum* SM1502^T among the most closely related species of genus *Flavobacterium* (Figs 1, S3, and S4). The phylogenomic tree was reconstructed using the TYGS (https://tygs.dsmz.de/) server. The TYGS phylogenomic tree also revealed that strains SaA2.12^T and LaA7.5^T formed a phyletic lineage with genus *Flavobacterium* (Fig. S5). Based on the 16S rRNA gene sequence similarities and the phylogenetic trees, it is suggested that strains SaA2.12^T and LaA7.5^T represent novel species within the genus *Flavobacterium*. Therefore based on 16S rRNA gene sequence similarity and phylogenetic trees, *F. arcticum* KCTC 42668^T, *F. suzhouense* KCTC 42107^T, *F. hauense* KCTC 32147^T and the genus type species *F. aquatile* JCM 20475^T were chosen as type strains for the polyphasic characterization of the novel isolates.

CHEMOTAXONOMY

Respiratory quinones were extracted and detected following the methods described by Komagata and Suzuki [34]. In brief, cells of strains SaA2.12^T and LaA7.5^T were cultured on MA for 4–5 days at 28 °C, harvested and freeze-dried. Subsequently, quinones were extracted from the freeze-dried cells (500 mg) with chloroform/methanol (2:1, v/v), applied onto TLC silica-gel plates, re-extracted with acetone and detected by LC-MS (Waters UPLC, Acquity UPLC HSS T3 C18 column). Polar lipids from novel strains SaA2.12^T, LaA7.5^T and two closest phylogenetic neighbours, *F. arcticum* KCTC 42668^T and *F. suzhouense* KCTC 42107^T, were extracted and identified

Table 1. Characteristics differentiating SaA2.12^T and LaA7.5^T from closely related species of the genus *Flavobacterium*

Strain: 1, SaA2.12^T; 2, LaA7.5^T; 3, *Flavobacterium arcticum* KCTC 42668^T; 4, *Flavobacterium suzhouense* KCTC 42107^T; 5, *Flavobacterium hauense* KCTC 32147^T; 6, *Flavobacterium aquatile* JCM 20475^T. All data were obtained in this study unless otherwise stated. +, Positive reaction; –, negative reaction; w, weak positive.

Characteristics	1	2	3	4	5	6
Isolation source	Surface water, Chaka Salt Lake	Shallow water sediment, Qinghai Lake	Arctic surface seawater	Farmland river sludge	Subsurface soil	Deep well
Temperature range for growth (°C) (optimum)	4–40 (28–30)	20–37 (28–35)	10–40 (25)	4–35 (28–30)	4–30 (28–30)	10–35 (25–28)
pH range for growth (optimum)	5.0–8.5 (5.0–7.5)	5.5–7.5 (5.5–7.0)	6.0–7.5 (7.0–7.5)	6.0–7.5 (7.0)	6.0–7.5 (7.0)	6.5–8.0 (7.0)
NaCl range for growth (% w/v) (optimum)	0–8.0 (0–5.5)	0–5.0 (0–2.5)	0–7.0 (0)	0–2.0 (0)	0–2.0 (0)	0–1.0 (0)
Gliding motility	+	+	+	–	–	–
Reduction of $NaNO_3$	+	+	+	–	–	+
H_2S production	+	+	+	–	–	–
Methyl red activity	+	–	–	–	–	–
Hydrolysis of:						
Starch	+	+	w	+	–	–
Tween 20	+	+	w	–	–	+
Tween 60	+	w	+	–	–	+
Tween 80	–	+	–	–	–	–
Casein	–	+	–	–	–	–
Enzyme activity (API ZYM):						
Lipase (C14)	+	–	–	–	–	–
Cystine arylamidase	–	+	+	+	–	+
Trypsin	+	–	+	+	–	–
Acid phosphatase	+	–	+	–	+	+
β-Glucosidase	–	+	–	+	–	–
API 20NE tests:						
Glucose fermentation	–	+	–	–	–	+
Arginine dihydrolase	+	–	–	–	–	+
Urease activity	+	–	–	–	–	–
Hydrolysis of gelatin	+	–	–	–	–	+
β-Galactosidase (PNPG)	+	–	–	w	–	+
Assimilation of mannose	+	–	–	–	–	–
Acid production from (API 50CH):						
L-Arabinose, sucrose	–	–	–	–	+	+
D-Galactose	–	–	–	+	+	+
Salicin	–	–	–	+	+	–
Trehalose	+	+	–	–	–	–
Gentiobiose	–	–	–	+	–	+

Continued

Table 1. Continued

Characteristics	1	2	3	4	5	6
Turanose	–	+	–	+	–	–
Potassium 2-ketogluconate	+	–	–	–	–	–
Potassium 5-ketogluconate	+	+	–	+	–	–
DNA G+C content (mol%)	38.0	37.8	34.9	38.4	43.9*	32.2

*Data from Dong *et al*. [46].

by two-dimensional TLC after Tindall [35]. For polar lipid analyses, cells of these strains were cultured on MA, harvested, freeze-dried and then extracted with chloroform/methanol (1:2, v/v). Spots of different polar lipids were detected by two-dimensional TLC on silica gel 60 F254 (Merck) plates (10×10 cm) by spraying with the appropriate detection reagents, the detailed procedure of which had been described previously [10, 36]. Furthermore, for analysis of the whole-cell fatty acids, the novel isolates and all studied reference strains were cultured on MA at 28 °C and their cellular biomass was harvested at the late-exponential phage of growth from MA. Subsequently, cellular fatty was extracted from the harvested cells and analysed according to the guidelines of the Microbial Identification System (MIDI) [37] using the TSBA6 database.

The predominant respiratory quinone of novel isolates SaA2.12^T and LaA7.5^T were menaquinone-6 (MK-6), which is the major quinone found in all members of *Flavobacterium* [18]. The polar lipid profile of strain SaA2.12^T was composed of phosphatidylethanolamine (PE), two unidentified aminolipids (AL1–2), two unidentified glycolipids (GL1–2), one unidentified phospholipid (PL) and five unidentified lipids (L1–5) (Fig. S6). In contrast, the polar lipid profile of strain LaA7.5^T contained PE, two unidentified aminolipid (AL1–2), one unidentified phospholipid (PL) and six unidentified lipids (L1–6) (Fig. S6). These two novel strains could be distinguished from each other by the presence/absence of glycolipids and the number of lipids. Moreover, these novel strains could be also separated from the closest type strains *F. arcticum* KCTC 42107^T and *F. suzhouense* KCTC32147^T by the presence of GLs, PL, the absence of aminophospholipids, and the number of AL and Ls (Figs S6 and S7). In addition, the presence of a large amount of PE in the polar lipid profiles of SaA2.12^T and LaA7.5^T was in the line with the characteristics of other *Flavobacterium* species [18]. Further chemotaxonomic results showed that the major fatty acids (above 10%) of the novel isolate SaA2.12^T were iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH and summed features 9 (iso-$C_{17:1}$ ω9*c* and/or $C_{16:0}$ 10-methyl) (Table 2). But for LaA7.5^T, the predominant fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Thus strain SaA2.12^T could be distinguished from LaA7.5^T by a different proportion of iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH, anteiso-$C_{15:0}$ and summed features 3 and 9 (Table 2). Compared with the fatty acid profile of the type strains, the profile of isolate SaA2.12^T showed relatively higher proportions of $C_{17:1}$ ω8*c*, anteiso-$C_{15:1}$ A, anteiso-$C_{17:1}$ ω9*c*, $C_{17:0}$ 2-OH, iso-$C_{15:0}$ 3-OH, iso-$C_{15:1}$ G, iso-$C_{16:0}$, iso-$C_{16:0}$ 3-OH, iso-$C_{16:1}$ H, iso-$C_{17:0}$ 3-OH, anteiso-$C_{15:0}$ and summed feature 9, and lower proportions of $C_{16:0}$, iso-$C_{15:0}$ and summed feature 3. On the other hand, the profile of novel strain LaA7.5^T showed significantly higher percentages of $C_{16:1}$ ω7*c*, iso-$C_{14:0}$, iso-$C_{17:0}$, iso-$C_{17:1}$ ω10*c*, anteiso–$C_{17:0}$, iso-$C_{15:0}$ (almost double than all other strains), anteiso-$C_{15:0}$ (almost seven times higher than all other strains) and summed feature 4 (iso-$C_{17:1}$ I or anteiso-$C_{17:1}$ B) and a lower percentage of almost all other fatty acids except those mentioned above (Table 2), which were remarkable differences between novel isolates LaA7.5^T and their closest neighbours. The chemotaxonomic results of the two isolates were consistent with other members of the genus *Flavobacterium* but numerous features in the polar lipid and fatty acid profiles described above could clearly differentiate them from other members of the genus *Flavobacterium*.

GENOME SEQUENCING AND ANALYSIS

To investigate the genomic relatedness of strain SaA2.12^T, LaA7.5^T and its closest neighbour *F. arcticum* KCTC 42668^T, genomic DNA was extracted with a genomic DNA extraction kit (Takara). Subsequently, genome sequencing was performed by Solexa PE150 sequencing technology with the HiSeq platform for strain SaA2.12^T and *F. arcticum* KCTC 42668^T (Beijing Genomics Institute, PR China), and LaA7.5^T (ShengTing Biotech Co., Ltd, PR China). SPAdes version 3.12.0 software was used for *de novo* assembly of the sequencing reads [38]. The genome of strains SaA2.12^T and LaA7.5^T were annotated by the NCBI prokaryotic genome annotation pipeline [39, 40] and the annotated genome sequences of SaA2.12^T and LaA7.5^T were deposited at GenBank/EMBL/DDBJ under the accession numbers SKBP00000000 and JAACYB000000000, respectively. Moreover, the genome sequence of *F. suzhouense* XIN-1^T (QQAR00000000), *F. subsaxonicum* DSM 21790^T (AUGP00000000) and the genus type strain *F. aquatile* LMG 4008^T=JCM 20475^T (JRHH00000000) were also obtained from the GenBank for comparison. The digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values between strains SaA2.12^T, LaA7.5^T and above-mentioned type strains were determined as described previously [36]. To

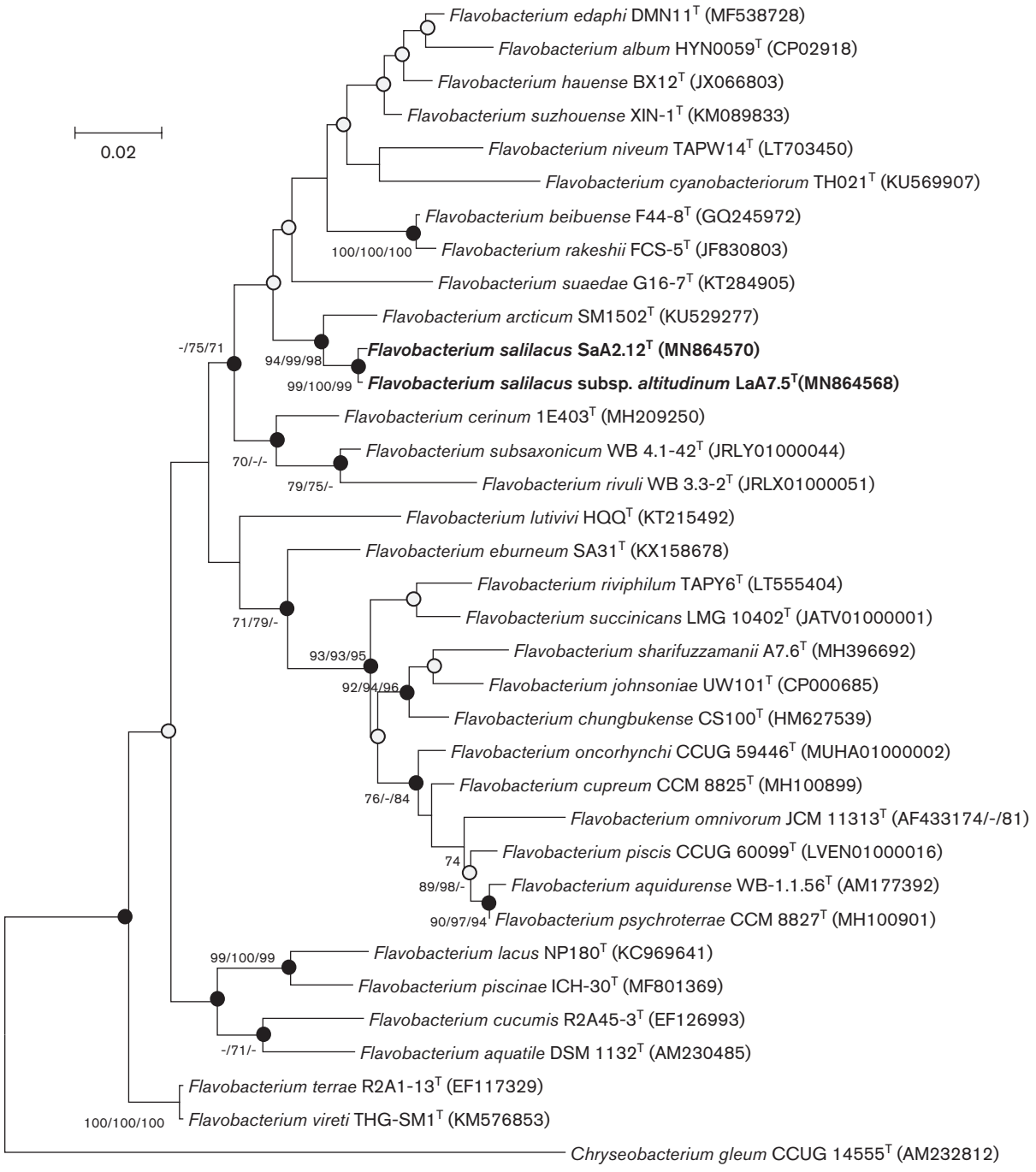

Fig. 1. Maximum-likelihood phylogenetic tree based on the 16S rRNA gene sequences, showing the phylogenetic position of strains SaA2.12^T and LaA7.5^T with the most closely related members. Bootstrap values were expressed as a percentage of 1000 replicates at the nodes, only those ≥70% are given at the branch points and were calculated using the maximum-likelihood, neighbour-joining, and maximum-parsimony methods, respectively. Filled and open circles indicate nodes recovered by all three tree-inferring methods or by two tree-inferring methods, respectively. *Chryseobacterium gleum* ATCC 35910^T (AM232812) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 2. Fatty acid composition (%) of novel isolates SaA2.12^T, LaA7.5^T and closely related *Flavobacterium* species

Strain: 1, SaA2.12^T; 2, LaA7.5^T; 3, *Flavobacterium arcticum* KCTC 42668^T; 4, *Flavobacterium suzhouense* KCTC 42107^T; 5, *Flavobacterium hauense* KCTC 32147^T; 6, *Flavobacterium aquatile* JCM 20475^T. All data were obtained during this study. –, Not found; TR, trace amount (<1%).

Fatty acids (%)	1	2	3	4	5	6
Saturated:						
$C_{10:0}$	1.2	TR	–	–	–	–
$C_{16:0}$	3.1	TR	1.1	8.2	7.4	3.2
Unsaturated:						
$C_{15:1}$ $\omega 6c$	1.6	–	1.3	2.1		TR
$C_{16:1}$ $\omega 7c$	–	2.9	–	–	–	–
$C_{17:1}$ $\omega 6c$	2.1	–	TR	TR	TR	4.3
$C_{17:1}$ $\omega 8c$	1.8	–	TR	TR	–	–
anteiso-$C_{15:1}$ A	1.3	–	TR	TR	–	–
anteiso-$C_{17:1}$ $\omega 9c$	1.8	–	–	–	TR	–
Hydroxy:						
$C_{17:0}$ 2-OH	1.3	TR	TR	TR	TR	–
Branched chain:						
iso-$C_{14:0}$	–	2.4	TR	TR	–	TR
iso-$C_{15:0}$	21.6	39.5	22.1	28.4	25.3	23.4
iso-$C_{15:0}$ 3-OH	4.9	TR	3.6	2.2	2.9	4.8
iso-$C_{15:1}$ G	11.2	TR	10.1	5.6	TR	6.3
iso-$C_{16:0}$	3.7	3.4	2.1	2.2	1.2	1.1
iso-$C_{16:0}$ 3-OH	3.0	TR	1.8	1.1	–	TR
iso-$C_{16:1}$ h	1.4	–	TR	TR	TR	–
iso-$C_{17:0}$	–	1.9	TR	TR	–	TR
iso-$C_{17:0}$ 3-OH	14.8	TR	8.2	11	7.9	2.3
iso-$C_{17:1}$ $\omega 10c$	–	2.1	–	–	–	–
anteiso-$C_{15:0}$	5.2	34.1	4.2	3.2	3.4	6.3
anteiso–$C_{17:0}$	–	4.0	–	–	–	–
Summed features:*						
3	4.8	TR	4.5	12.1	17.4	5.9
4	–	1.7	–	5.7	2.7	–
9	13.3	–	–	4.2	3.3	3.5

*Summed features are groups of two or three fatty acids that could not be separated by the MIDI system. Summed feature 3 is composed of $C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$; summed feature 4 is composed of iso-$C_{17:1}$ I or anteiso-$C_{17:1}$ B; summed feature 9 is composed of iso-$C_{17:1}$ $\omega 9c$ and/or $C_{16:0}$ 10-methyl.

mine the potential of producing secondary metabolites, the genomes of the novel strains SaA2.12^T, LaA7.5^T and closest neighbours *F. arcticum* KCTC 42668^T, *F. suzhouense* XIN-1^T, *F. subsaxonicum* DSM 21790^T were analysed with antiSMASH (https://antismash.secondarymetabolites.org) version 5.0 beta [41].

The G+C content of the genomic DNA of both strains SaA2.12^T and LaA7.5^T were 38.0 and 37.8 mol%, respectively (Table 1). This G+C content values were within the range of 30–52 mol% reported for the members of the genus *Flavobacterium* [7]. The genome annotation revealed 2715 coding DNA sequences (CDSs), 50 RNA genes, 46 tRNAs and four ncRNAs within strain SaA2.12^T and 2768 CDSs, 59 RNA genes, 50 tRNAs and four ncRNAs in strain LaA7.5^T. To exactly clarify the taxonomic position of these two strains, the genome-based dDDH value and ANI values were calculated in this study. The dDDH and ANI values between strain SaA2.12^T and the phylogenetic neighbours mentioned above ranged from 18.3–25.3 and 69.8–82.8%, respectively (Table 3). Concordantly, 18.4–25.2% for dDDH and 70.0–82.8% for ANI was observed between novel strain LaA7.5^T and the above-mentioned type strains, which are almost similar to the values between SaA2.12^T and the type strains (Table 3). These dDDH and ANI values were significantly below the 70% cut-off value for dDDH and the 95–96% cut-off value for ANI proposed for the delineation of bacterial species [33, 42, 43]. Therefore, the dDDH and ANI results strongly suggest that strains SaA2.12^T and LaA7.5^T represent two novel species of the genus *Flavobacterium*. In addition, the genome-based dDDH and ANI values between the two novel strains SaA2.12^T and LaA7.5^T were 75.9 and 97.2%, respectively. These values were slightly higher than the above mentioned cut-off values (70% for dDDH and 95% for ANI) proposed for the delineation of bacterial species [33, 42, 43], indicating that they were not different species of the genus *Flavobacterium*. While there is no ANI cut-off value to define subspecies, a <79–80% dDDH value is used as a standard threshold value for bacterial subspecies delineation [44]. Hence, both ANI and dDDH analyses lead us to consider that the isolate SaA2.12^T represents a novel *Flavobacterium* species and LaA7.5^T is a novel subspecies of SaA2.12^T, which is the first description of any subspecies of the genus *Flavobacterium*.

Genome mining with antiSMASH showed two terpene biosynthetic gene clusters within strain SaA2.12^T and LaA7.5^T (Table 4). Similarly, the closest neighbour, *F. arcticum* KCTC42668^T, had two gene clusters associated with the biosynthesis of terpenes; besides these two gene clusters, *F. suzhouense* XIN-1^T showed additional one polyketide (PK) gene cluster; and *F. subsaxonicum* DSM 21790^T showed four biosynthetic gene clusters, including two terpenes, one type I polyketide and one type III polyketide (Table 4). The flexirubin-type gene cluster was absent in both novel strains while carotenoid-type gene clusters were present. In our experiment, we also found that these novel isolates produce carotenoids (zeaxanthin) but do not produce flexirubin-type pigments. So genome mining results also support the phenotypic results but improved genome alignments are required

Table 3. The digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values between strains SaA2.12^T, LaA7.5^T and their closely related species of the genus *Flavobacterium*

All data were obtained in this study unless otherwise stated.

Member of genus *Flavobacterium*	GenBank accession no.	Genome size (bp)	ANI (%)	DDH (%)
SaA2.12^T vs. other strains				
SaA2.12^T	SKBP00000000	3012773	–	–
LaA7.5^T	JAACYB000000000	3185588	97.2	75.9
Flavobacterium arcticum KCTC 42668^T	SKBO00000000	2945587	82.8	25.3
Flavobacterium suzhouense XIN-1^T	QQAR00000000*	3829887	73.4	18.5
Flavobacterium subsaxonicum DSM 21790^T	AUGP00000000*	4638566	73.2	18.3
Flavobacterium aquatile JCM 20475^T	JRHH00000000*	3490856	69.8	18.4
LaA7.5 ^{T} vs. other strains				
Flavobacterium arcticum KCTC 42668^T	SKBO00000000	2945587	82.8	25.2
Flavobacterium suzhouense XIN-1^T	QQAR00000000*	3829887	73.4	18.6
Flavobacterium subsaxonicum DSM 21790^T	AUGP00000000*	4638566	73.3	18.4
Flavobacterium aquatile JCM 20475^T	JRHH00000000*	3490856	70.0	18.6

*Retrieved from NCBI GenBank.

to achieve a better understanding of the novel isolates and its closest type strains.

The unique morphological, cultural, phenotypical, phylogenetic, chemotaxonomic and genetic data described above clarify that strains SaA2.12^T and LaA7.5^T are not clones of each other but are new members of the genus *Flavobacterium*. Therefore, we propose that isolate SaA2.12^T should be considered as a novel species of the genus *Flavobacterium* with the name *Flavobacterium salilacus* sp. nov., the first description of any *Flavobacterium* species that have been isolated from a hypersaline environment; whereas strain LaA7.5^T be recognized as the first description of a subspecies of *Flavobacterium salilacus* sp. nov., with the name *Flavobacterium salilacus* subsp. *altitudinum* subsp. nov. According to Rule 40d of the International Code of Nomenclature of Prokaryotes [45], the description of a novel subspecies *Flavobacterium salilacus* subsp. *altitudinum* excluding the type strain of the species *Flavobacterium salilacus* automatically creates another subspecies *Flavobacterium salilacus* subsp. *salilacus* subsp. nov., for which the type stain is SaA2.12^T.

Table 4. The predicted secondary metabolites (SM) gene clusters of novel isolates SaA2.12^T, LaA7.5^T and their closely related *Flavobacterium* species

Strain: 1, SaA2.12^T; 2, LaA7.5^T; 3, *Flavobacterium arcticum* KCTC 42668^T; 4, *Flavobacterium suzhouense* KCTC 42107^T; 5, *Flavobacterium subsaxonicum* DSM 21790^T. +, Gene cluster present; –, gene cluster absent.

Type of SM gene cluster	1	2	3	4	5
Terpene:					
Carotenoid type terpene	+	+	+	+	+
Unknown	+	+	+	+	+
Polyketides:					
Flexirubin type PK	–	–	–	+	+
Unknown type III PKS	–	–	–	–	+

DESCRIPTION OF *FLAVOBACTERIUM SALILACUS* SP. NOV.

Flavobacterium salilacus (sa.li.la′cus. L. masc. n. sal salt; L. masc. n. lacus lake; N.L. gen. n. *salilacus* of a salt lake).

Cells of strain SaA2.12^T are Gram-stain-negative, aerobic, motile-by-gliding, non-flagellated, non-spore forming, rod-shaped, 0.5–0.6 µm wide and 1.2–1.4 µm long (Fig. S1). Colonies are yellow in colour, circular, raised, have entire margins and are 2–3 mm in diameter on MA after 3–4 days of incubation. Cells grow on MA, TSA, R2A agar and NA, but weakly on LB and TYS agar plates. Growth occurs at 4–40 °C (optimum, 28–30 °C), at pH 5.0–8.5 (optimum, pH 5.0–7.5) and at 0–8.0% (w/v) NaCl (optimum, 0–5.5%). The flexirubin-type pigment is not produced, Congo red is not absorbed but carotenoids (zeaxanthin) are produced. Cells can hydrolyse starch, Tween 20 and Tween 60, but unable to hydrolyse casein, CM-cellulose, hypoxanthine, xanthine, Tween 40, Tween 80, filter paper (crystalline cellulose) or tyrosine; cells are positive for oxidase-activity, catalase activity, nitrate reduction, H_2S production and methyl red tests; negative for the Voges–Proskauer test and unable to grow in the anaerobic condition. API ZYM kit results show that cells are positive for

alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activity, weakly positive for esterase (C4) activity, and negative for all other activities except those mentioned above. API 20NE results are negative for all except arginine dihydrolase, urease activity, PNPG (β-galactosidase) activity, hydrolysis of β-glucosidase (aesculin), gelatin and assimilation of mannose. In API 50CH kit tests, the cells produce acid only from D-glucose, D-mannose, amygdalin, aesculin, cellobiose, maltose, trehalose, starch, glycogen and potassium 5-ketogluconate. The major fatty acids are iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH and summed feature 9 (iso-$C_{17:1}$ ω9*c* and/or $C_{16:0}$ 10-methyl). The major respiratory quinone is MK-6 and the major polar lipids are PE, PL, two ALs, two GLs and five Ls. The DNA G+C content is 38.0 mol%.

The type strain is SaA2.12^T (KCTC 72220^T=MCCC 1K03618^T), isolated from the surface water of the hypersaline lake, Chaka Salt Lake, Qinghai Province, PR China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequences are MN864570 and SKBP00000000, respectively.

DESCRIPTION OF *FLAVOBACTERIUM SALILACUS* SUBSP. *ALTITUDINUM* SUBSP. NOV.

Flavobacterium salilacus subsp. *altitudinum* (al.ti.tu'di.num. L. gen. pl. n. *altitudinum* of high places).

Cells are Gram-stain-negative, aerobic, motile-by-gliding, non-flagellated, non-spore forming, rod-shaped, approximately 0.4–0.6 µm wide and 0.8–1.5 µm long (Fig. S1). Colonies are circular, raised, have entire margins, 1–2 mm and yellow in colour within 3–4 days of incubation on MA. Cells grow on MA, TSA, R2A agar and NA but weakly on LB and TYS agar plates. Growth occurs at 20–37 °C (optimum, 28–35 °C), at pH 5.5–7.5 (optimum, pH 5.0–7.0) and at 0–5.0% (w/v) NaCl (optimum, 0–2.5%). Carotenoids (zeaxanthin) are produced, but flexirubin-type pigment is not produced and Congo red is not absorbed. Cells are able to hydrolyse starch, casine, Tween 20 and Tween 80, weakly hydrolyse Tween 60, but unable to hydrolyse CM-cellulose, hypoxanthine, xanthine, Tween 40, filter paper (crystalline cellulose) or tyrosine. Cells are positive for oxidase-activity, catalase activity, nitrate reduction and H_2S production, and negative for methyl red and Voges–Proskauer tests. API ZYM kit results show that cells are positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activity, weakly positive for esterase (C4) activity, and negative for all other activities. In the API 20NE kit system, cells are negative in all tests except for fermentation of glucose and hydrolysis of β-glucosidase (aesculin). For API 50CH kits, the cells produce acid only from D-glucose, D-mannose, amygdalin, aesculin, cellobiose, maltose, trehalose, starch, glycogen, turanose and potassium 5-ketogluconate. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The major respiratory quinone is MK-6 and the major polar lipids are PE, PL, two ALs and six Ls. The DNA G+C content is 37.8 mol%.

The type strain is LaA7.5^T (KCTC 72806^T=MCCC 1K04372^T), isolated from shallow water sediment collected from Qinghai Lake, Qinghai Province, PR China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequences are MN864568 and JAACYB000000000, respectively.

DESCRIPTION OF *FLAVOBACTERIUM SALILACUS* SUBSP. *SALILACUS* SUBSP. NOV.

Flavobacterium salilacus subsp. *salilacus* (sa.li.la'cus. L. masc. n. sal salt; L. masc. n. lacus lake; N.L. gen. n. *salilacus* of a salt lake).

The description of this subspecies follows the species description of *Flavobacterium salilacus* sp. nov. However, this subspecies can be distinguished from *Flavobacterium salilacus* subsp. *altitudinum* subsp. nov. based on: different isolation source and different geographic location; growth at high NaCl concentration (can grow at 0–8.0% NaCl, w/v); methyl red activity; inability to hydrolyse Tween 80 and casine; positive activity for lipase (C14), trypsin and acid phosphatase; negative activity for cystine arylamidase and β-glucosidase; positive for arginine dihydrolase, urease activity, PNPG (β-galactosidase) activity and gelatin; assimilation of mannose; inability to glucose fermentation; and ability to produce acid from potassium 2-ketogluconate and inability from turanose. Moreover, the presence of higher proportions of iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH, summed feature 3 and summed feature 9, and lower proportions of iso-$C_{15:0}$ and anteiso-$C_{15:0}$ in the fatty acid profile and the presence of two GLs in the polar lipid profile clearly differentiates *Flavobacterium salilacus* subsp. *salilacus* from *Flavobacterium salilacus* subsp. *altitudinum*.

The type strain is SaA2.12^T (KCTC 72220^T=MCCC 1K03618^T), isolated from the surface water of a hypersaline lake, Chaka Salt Lake, Qinghai Province, PR China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and genome sequences are MN864570 and SKBP00000000, respectively.

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

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