Description and genome analysis of Aoguangibacterium sediminis gen. nov., sp. nov., a noval denitrifying and carbon-fixing bacterium isolated from marine sediment

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Research Article

Keywords: a novel genus, Flavobacteriaceae, marine sediment, taxonomy, bacterium

Posted Date: May 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1648654/v1

Abstract

A Gram-stain-negative, non-motile, aerobic, red colony, mesophilic, and slightly halotolerant bacteria with carbon fixation and denitrification, designated strain D16^T, was separated from intertidal surface sediments from Xiaoshi Island, Weihai, China. The strain was able to grow at 10–43°C, pH 5.5–9.5 and in 0–5% (w/v) NaCl. Optimum growth conditions of the strain were at pH 7.0, 30–33°C and 1% (w/v) NaCl. The predominant respiratory quinone, major fatty acids and polar lipids of the strain were (MK-6), (iso-$C_{15:0}$, 32.0%, $C_{16:0}$, 10.5%) and (DPG□PE□AGL□ GL□ AL□PGS), respectively. Phylogenetic analysis based on 16S rRNA gene sequences and genomes indicated that strain D16^T was the closest to genera *Eudoraea*, *Poritiphilus, Muriicola*, *Robiginitalea* and *Zeaxanthinibacter*, sharing 92–93% 16S rRNA gene sequence similarity with the representative of these genera. The draft genome of strain D16^T composed 3,225,638 bp, and the G + C content was 42.8 mol %. The ANI, dDDH, AAI values of strain D16^T compared to similar genera ranged from 67.3 to 70.2%, 16.2 to 19.1% and 68.3 to 70.1%, respectively, below the threshold for recommended species division of ANI 95–96%, dDDH 70% and AAI 95%. Furthermore, POCP values between strain D16^T and similar genera were less than 50.0%, consistent with the description of a new genus. Based on the results of phylogenetic, phenotypic, genotypic, c*hem*otaxonomic analyses and comparative analysis of metabolic pathway, environmental distribution and characterization between in strain D16^T and other relative genera of family *Flavobacteriaceae*, it is concluded that strain D16^T represents a novel species of a noval genus of the family *Flavobacteriaceae*, for which the name *Aoguangibacterium sediminis* gen.nov., sp. is proposed. The type strain is D16^T (= MCCC 1H00463^T = KCTC 82746^T).

Introduction

Studies have shown that marine microorganisms play an important role in the global carbon, nitrogen cycle, (Li et al. 2018; Cai et al. 2021) and they are responsible for the remineralization of organic matter and the transfer of nutrients and energy to higher nutrient levels in the ocean (Worden et al. 2015). Carbon is one of the most important elements of all living substances, and it is also the most abundant element on earth. Carbon fixation, calcification and heterotrophy of marine microorganisms are the three elements involved in the carbon cycle of the marine environment(Cavicchioli et al. 2019). Nitrogen, as one of the essential elements of life activity undertaker protein, has immeasurable significance. Marine microbial communities perform complex bioc*hem*ical transformations that drive the nitrogen cycle, including nitrogen fixation, assimilation, nitrification, and nitrogen loss processes (Hutchins and Capone 2022).

The *Flavobacteriaceae* family, belonging to the biggest family of the class *Flavobacteriia* in the phylum *Bacteroidota*, was first described in 1996 (Bernardet et al. 1996). Over the past 25 years, the number of genera described in the *Flavobacteriaceae* family has increased from 10 to 178 (Parte 2018). The taxonomy of the family has undergone major changes with new members continuing to join. Most members of the family are short to moderately long rods, Gram-stain-negative that are not able to degrade crystalline cellulose. High levels of iso-$C_{15:0}$ and i-$C_{17:0}$ 3-OH and the DNA G + C contents range from 27 to 45 mol % were found in members of the family, according to the revised description of family *Flavobacteriaceae* by Bernardet *et al.*(Bernardet et al. 2002). *Flavobacteriaceae* were common in terrestrial and freshwater environments and dominated marine habitats. In the *Flavobacteriaceae* family, bacteria isolated from marine sources are widely distributed in all clades, but a large proportion belongs to marine clades. Marine *flavobacterium* has been found to live freely in the water column or attach to debris. Their lifestyle includes colonization of surfaces by algae but is also closely associated with invertebrates, for instance, sponges, corals and echinoderms. Most members are not autotrophic and can degrade macromolecules, such as complex polysaccharides and proteins, whereas degradation is greatly different. Therefore, the family plays a key role in the material cycle of the marine environment(Williams et al. 2013).

In this study, a noval denitrifying and carbon-fixing bacterium (designated D16^T), separated from intertidal surface sediments in Weihai, Shandong, PR China, was determined its taxonomic position using polyphasic classification including phenotypic, phylogenetic analysis, c*hem*otaxonomic characterization, genomic comparative analysis and environmental distribution, which was characterized as Gram-stain-negative, mesophilic, non-motile, red, aerobic, non-spore-forming, denitrifying, carbon-fixing and slightly halotolerant. Both autotrophic and heterotrophic lifestyle were found in the strain D16^T. Data from this study propose that the strain did not belong to any of the existing genus of family *Flavobacteriaceae*. Thus, it was recommended as a new genus and species, *Aoguangibacterium sediminis* gen. nov., sp. nov., within the *Flavobacteriaceae* family.

Materials And Methods

Sample collection and strains isolation

The strain D16^T, separated from a marine sediment sample, which was collected from the intertidal zone of Xiaoshi Island, Weihai, China (37°34'32" N, 122°9'19" E), in July 2020. The sample preserved at four °C was used for bacterial isolation. The isolation was done using the traditional Dilution Plating Procedure on solidified medium containing 0.5 g (w/w) peptone and 0.1 g (w/w) yeast extract perlites, and then cultivation at 30°C for a week. Strain D16^T, after being isolated, was stored using glycerol tube cryopreservation. The type strains *Eudoraea adriatica* DSM 19308^T, *Poritiphilus flavus* R33^T, *Muriicola jejuensis* EM44^T, *Robiginitalea biformata* HTCC 2501^T and *Zeaxanthinibacter enoshimensis* TD-ZE3^T (preserve in our lab), as reference strains of strain D16^T, were chosen and grown on MA(Marine agar) medium at optimum temperature, respectively in this study.

16S rRNA gene sequencing and phylogenetic analysis

16S rRNA gene sequence of D16^T was acquired using a published procedure (Sanger et al. 1977; Liu et al. 2014). 16S rRNA gene pairwise similarities were aligned using the NCBI nucleotide database[2], the EzTaxon-e server[3] (Yoon et al. 2017) and SILVA SSU databases 138.1(Quast et al. 2013). After performing multiple alignment of the sequences using MUSCLE, phylogenetic analysis was accomplished using MEGA 11 (Tamura et al. 2021). The phylogenetic trees based on aligned sequences were reconstructed using the neighbor-joining (Saitou and Nei 1987), maximum likelihood (Felsenstein 1981) and maximum-parsimony (Kannan and Wheeler 2012; Herbst and Fischer 2017) algorithms in the software package MEGA version 11. The Kimura two-parameter model estimated the phylogenetic distance matrices (Nishimaki and Sato 2019). Genome sequences of strain D16^T and closely related genera were aligned by

GTDB-Tk(Chaumeil et al. 2019), and the phylogenetic tree based on genomes was reconstructed in GTDB-Tk. The phylogenetic tree was displayed in itol[5] (Letunic and Bork 2021).

Genomic characteristics and Comparative Genomic Analysis

The genomic DNA of the strain D16^T was extracted using a genomic DNA extraction kit (Takara). The genome sequencing of strain D16^T was performed by Beijing Novogene Bioinformatics Technology using the Illumina HiSeq PE150 platform.

The genome sequences of closely related genera were downloaded from NCBI. The genes involved in metabolic pathways of the strain D16^T and close relatives were analyzed using the RAST(Overbeek et al. 2014), the KEGG (Aramaki et al. 2020) and the Prokka rapid prokaryotic genome annotation (Seemann 2014). CAZymes were annotated against dbCAN database version 6.0 based on HMMER search (HMMER 3.0b) (Zhang et al. 2018). Secondary metabolite BGCs were identified by the online server antiSMASH 6.0 with “relaxed” detection strictness. The average amino acid sequence identity (AAI)the average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values between strain D16^T and close relatives were calculated using the AAI calculator ANI calculator in the EzBioCloud database and the Genome-to-Genome Distance Calculator[7] (Lachance et al. 2020; Kim et al. 2021; Meier-Kolthoff et al. 2022) respectively. In addition, the percentage of conserved proteins (POCP), recommended as a new genus threshold, between strain D16^T and relative genera were calculated using the BLASTP program (Qin et al. 2014). Scatter plots and taxonomic profiles were visualized using the AAI-profiler online server[9](Medlar et al. 2018).

Phenotypic, Physiological, and Bio*chem*ical Characteristics

The shape and size of cells were tested by scanning electron microscope (SEM, Nova NanoSEM450, FEI). Cells were observed using an optical microscope after grown in MA broth at 33°C for 72 hours and standard Gram stained. The gliding motility test was performed on MA broth supplemented with 0.2% agar using a phase-contrast optical microscope. Growth at different temperatures (0, 4, 8, 12, 16, 20, 24, 28, 30, 33, 35 and 40°C) was examined in MA broth. The pH range (4.0–10.0, at 0.5 pH unit intervals) for growth was determined in MA broth at 33°C. The pH of the medium was adjusted with 1 M NaOH or 1 M HCl after being added into suitable MES, MOPS, Tris and CHES, respectively. The NaCl concentration range for growth (0, 0.5, 1–20 % at 1 % intervals, w/v) was detected in artificial marine agar 2216 (MA) compounded with artificial seawater without NaCl, 0.1/0.5 % yeast extract/tryptone, and 0–20% NaCl.

The strain D16^T, growth under microaerobic (85% N_2, 10% CO_2, and 5% O_2) and anaerobic (80% N_2, 10% CO_2, and 10% H_2) surroundings, was tested after being cultured for 14 days on MA in microaerobic and anaerobic condition with and absent 0.1% (w/v) KNO_3. Nitrification, degradation of agar, starch, casein, CM-cellulose, Tweens (20, 40, 60 and 80) and alginate, oxidase and catalase activities were tested as described by Kamlage et al. (Kamlage 1996). Additional physiological, bio*chem*ical and enzymatic activity tests of strain D16^T and similar strains were performed using the API 50 CH, API 20E and API ZYM identification systems (bioMérieux) and the Biolog GEN III identification system according to the manufacturers’ manuals under the same laboratory condition. Antibiotic susceptibility was determined according to the published susceptibility test protocol (Ahmed et al. 1966).

C*hem*otaxonomic Characterization

For the analysis of the c*hem*otaxonomic features, strain D16^T has grown in marine broth 2216 medium at 33°C for 72 hours while the five related type strains, *Eudoraea adriatica* DSM 19308^T, *Poritiphilus flavus* R33^T, *Muriicola jejuensis* EM44^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T and *Robiginitalea biformata* HTCC 2501^T were collected during the exponential growth phase and ultra-low temperature vacuum freeze drying after cultivated in marine broth 2216 at 33°C for 72 hours. The fatty acids in cells were extracted following the standard protocol of the Microbial Identification System (MIDI; Microbial ID, version 6.3B). And component analysis of fatty acids was performed by Gas Chromatography (Agilent Technologies 6890). The respiratory quinone of strain D16^T was measured by HPLC after extraction with a chloroform/methanol (2:1, v/v) mixture and separated by TLC as described previously (Komagata and Suzuki 1988). Polar lipids were separated by two-dimensional silica gel TLC (Merck Kieselgel 60 F254) and extracted from lyophilized cells of strain D16^T and four related type strains (Minnikin et al. 1984).

Results And Discussion

Phylogenetic characteristics

The 16S rRNA gene sequence (1511 bp; GenBank accession number MZ695036) showed that the closely related species of strain D16^T were genus or members of the genera *Eudoraea adriatica* DSM 19308^T (93.0 %), *Poritiphilus flavus* R33^T (92.5 %), *Muriicola* (91.9-92.4 %), *Zeaxanthinibacter* (91.7-92.3 %), *Robiginitalea* (91.9-92.3 %), *Cellulophaga* (91.2-91.7), *Maribacter* (90.9-92.0 %) and *Arenibacter* (90.9-91.5 %) in the family *Flavobacteriaceae*. All the similarities between strain D16^T and closely related genera were lower than 94 %, which was considered a reasonable lower cut-off window for describing a new genus (Yarza et al. 2014; Varghese et al. 2015; Konstantinidis et al. 2017; Johnson et al. 2019). The neighbor-joining phylogenetic tree (Supplementary Figure 1) showed that strain D16^T belonged to the clade represented by the closely related genera listed above. Still, strain D16^T formed a single lineage in this clade with a high bootstrap value (97 %) (Fig. 1). In the maximum-likelihood tree (Supplementary Figure 2) based on single-copy orthologous clusters (concatenated protein sequences), like in neighbor-joining, maximum-likelihood and maximum-parsimony trees (Supplementary Figure 3) based on 16S rRNA gene sequences, strain D16^T formed a separate branch, at the root of the branch formed by member of the genus *Flavobacterium*, within the cluster of the family *Flavobacteriaceae*, supporting its affiliation with a novel genus. A phylogenomic tree built based on genome sequences strain D16^T and relative genera showed the same phylogenetic position of strain D16^T (Supplementary Figure 4).

Genome composition and Genomes comparison

The genome size of strain D16^T was 3,225,638 bp. According to the NCBI PGAP, strain D16^T was predicted 2,981 genes in the genome, including 2,916 protein-coding genes. The amino acid sequence of strain D16^T and close relatives were analyzed by KEGG and 1374 genes (46.3%) could be assigned a putative function (Supplementary Figure 5). Strain D16^T contains 184 strain-specific genes. The numbers of gene functional categories comparison of strain D16^T *and Eudoraea adriatica* DSM 19308^T, *Robiginitalea biformata* HTCC2501^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T, *Cellulophaga tyrosinoxydans* EM41^T and *Muriicola jejuensis* EM44^T were shown in Supplementary Figure 6.

The genomic DNA G+C content of strain D16^T was 42.8 mol %, which was in the range (37.8–55.3 mol%) of DNA G+C contents in the family *Flavobacteriaceae* (Table 1). The ANI, dDDH, AAI values of strain D16^T compared to similar genera ranged from 67.3 to 70.2 %, 16.2 to 19.1 % and 68.3 to 70.1 %, respectively (Table 2), below the threshold for recommended species division of ANI 95–96%, dDDH 70% and AAI 95% (Lachance et al. 2020; Kim et al. 2021; Meier-Kolthoff et al. 2022). The heat map results of DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) values of genomes between D16^T and related strains are presented in Supplementary Figure 7. The AAI-profiler comparison of amino acids of D16^T results suggested that the closest sequenced proteome was from *Eudoraea adriatica* DSM 19308^T, with 65% matched fraction and 68.32% average amino acid identity values (Supplementary Figure 8). These data indicated that strain D16^T represents a novel species. Furthermore, POCP values between strain D16^T and similar genera were less than 50.0%, consistent with the description of a new genus (Qin et al. 2014). Further supported that strain D16^T was a novel genus of the family *Flavobacteriaceae*.

Table 1. Genome statistics of strain D16^T and the related genera in family *Flavobacteriaceae*.

Strains: 1, D16^T; 2, *Eudoraea adriatica* DSM 19308^T(Alain et al. 2008); 3, *Zeaxanthinibacter enoshimensis* TD-ZE3^T(Asker et al. 2007); 4, *Robiginitalea biformata* HTCC2501^T(JC and SJ 2004); 5, *Muriicola jejuensis* EM44^T(Kahng et al. 2010); 6, *Cellulophaga tyrosinoxydans* EM41^T(Kahng et al. 2009); 7, *Poritiphilus flavus* R33^T(Wang et al. 2020)

characteristics	1	2	3	4	5	6	7
Size of genome (mb)	3.1	3.91	3.34	3.53	3.31	3.56	4.
N50 value(bp)	281,706	562,897	709,771	3,530,383	1,788,650	377,537	55
G+C content (mol%)	37.8	38.3	46.4	55.3	48.3	33.2	44
Contigs(no.)	20	22	13	1	26	18	19
Annotation by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP)							
Genes(no.)	2981	3476	2953	3070	2921	3166	4,
Protein-coding genes (no.)	2916	3401	2897	3131	2994	3106	41
Gene specific to genome	183	79	48	64	34	120	56
tRNA(no.)	40	36	38	41	39	33	3
rRNA(no.)	3	9	3	6	3	6	38
ncRNAs (no.)	4	4	4	4	4	4	4
DDBJ/ENA/GenBank accession number	JAJTJC000000000	KB907546	NZ_SNYI00000000	NC_013222.1	NZ_JAABOP000000000.1	NZ_FWXO01000000	N

Table 2 AAI, ANI, POCP, DDH values among strain D16^T and relative genera of the family *Flavobacteriaceae*

Strain	Accession no.	AAI/%	ANI/%	POCP/%	DDH/%	Reference
D16^T	JAJTJC000000000	100.0	100.0	100.0	100.0	This study
Robiginitalea biformata HTCC2501^T	KB907546	71.4	69.6	71.4	16.5	(JC and SJ, 2004)
Muriicola jejuensis EM44^T	NZ_SNYI00000000	70.1	68.9	67.9	17.0	(Kahng *et al*., 2010)
Eudoraea adriatica DSM 19308^T	NC_013222.1	68.3	68.4	66.3	19.2	(Alain *et al*., 2008)
Zeaxanthinibacter enoshimensis TD-ZE3^T	NZ_JAABOP000000000.1	70.4	69.6	75.1	16.8	(Asker *et al*., 2007)
Cellulophaga tyrosinoxydans EM41^T	NZ_FWXO01000000	68.7	68.4	62.4	16.2	(Kahng *et al*., 2009)
Saonia flava DSM 29762^T	NZ_JAATJJ000000000	70.1	69.2	62.6	16.8	(Fagervold *et al*., 2017)
Maribacter polysiphoniae KCTC 22021^T	NZ_JACWLN000000000	70.3	69.3	22.7	16.8	(Nedashkovskaya *et al*., 2007)
Robiginitalea sediminis O458^T	NZ_NGNR00000000	68.2	68.4	71.3	16.4	(Zhang *et al*., 2018b)
Robiginitalea myxolifaciens DSM 21019^T	NZ_FOYQ00000000	67.5	68.2	69.2	18.0	(Manh *et al*., 2008)
Maribacter aquivivus DSM 16478^T	NZ_FQZX00000000	69.2	68.0	53.2	16.6	(Nedashkovskaya *et al*., 2004)
Maribacter algicola PoM-212^T	NZ_QUSX00000000	68.7	68.5	62.3	16.9	(Khan *et al*., 2020)
Arenibacter troitsensis DSM 19835^T	NZ_FXAO00000000	69.3	68.9	55.4	17.3	(Nedashkovskaya *et al*., 2003)
Maribacter luteus RZ05^T	NZ_WKJH01000030	69.9	69.2	59.6	17.7	(Liu *et al*., 2020)
Maribacter arenosus CAU 1321^T	NZ_JABTCG010000000	70.0	69.0	65.0	18.0	(Thongphrom *et al*., 2016)
Maribacter aurantiacus KCTC 52409^T	NZ_VBUK00000000	68.9	68.5	60.9	17.1	(Khan *et al*., 2020)
Maribacter orientalis DSM 16471^T	NZ_FNZN00000000	68.1	68.0	61.2	17.2	(Nedashkovskaya *et al*., 2004)
Cellulophaga algicola DSM 14237^T	NC_014934	68.1	68.1	51.0	18.4	(Bowman, 2000)
Maribacter flavus KCTC 42508^T	NZ_VUOE00000000	69.0	68.7	61.3	17.3	(Tang *et al*., 2015)
Maribacter vaceletii DSM 25230^T	NZ_RBIQ00000000	68.9	68.9	57.1	16.3	(Jackson *et al*., 2015)
Arenibacter aquaticus GUO^T	NZ_RQPJ00000000	69.5	68.9	54.4	18.1	(Guo *et al*., 2020)
Arenibacter nanhaiticus CGMCC 1.8863^T	NZ_SNZW00000000	68.0	68.2	60.4	17.5	(Sun *et al*., 2010)
Maribacter caenipelagi CECT 8455^T	NZ_FNBD00000000	68.4	68.3	41.8	16.5	(Jung *et al*., 2014)
Cellulophaga baltica DSM 24729^T	PRJNA599484	71.2	69.8	61.2	17.5	(Johansen *et al*., 1999)
Poritiphilus flavus R33^T	GCA_009901585.1	71.1	68.3	61.2	17.5	(Wang et al., 2020)

Genome Function prediction

The RAST annotation indicated that gene functions of strain D16^T covered all aspects of cellular metabolism, with the most diverse gene types and number associated with biological process (917 metabolic pathways). Statistics of metabolic function of strain D16^T are shown in Supplementary Figure 9. About 110 carbon metabolism related pathways was annotated in genome of strain D16^T, which also includes relavite genes and enzymes of CO_2 fixation, such as Ss RBCS RBCl ClCP CA. There are13 metabolism pathways associated with nitrogen metabolism was annotated in genome of D16^T, which including denitrification pathway, denitrification-related genes, such as *nar*H and *nar*I, which was consistent with the results of anaerobic experiments. Furthermore, 33 metabolism pathways associated with cellular respiration were found in the strain D16^T. [NiFe], One of the special key enzymes, also is closely related with carbon fixation. the strain D16^T may be involved in carbon and nitrogen cycling in marine sediments, predict from the above analysis. CO_2 fixation denitrification and [*NiFe*] was also found in other relative genera of family *Flavobacteriaceae* [Supplementary table 2].

Metabolic pathway comparison

Some complete metabolic pathways have been found in strain D16^T and close relatives (Supplementary Figure 10). For Fatty acid biosynthesis, beta-Oxidation, acyl-CoA synthesis, biosynthesis of Inosine monophosphate, adenine ribonucleotide, guanine ribonucleotide and pyrimidine deoxyribonuleotide. These pathways were found in all these strains. Conversely, reductive pentose phosphate cycle, uridine monophosphate biosynthesis, ethylene biosynthesis and urea cycle present in all these strains were found to be incomplete.

Some metabolic pathways exhibited striking differences among these *Flavobacteriaceae* strains (Figure 2). For instance, Strain D16^T, *Maribacter polysiphoniae* KCTC 22021^T and *Arenibacter troitsensis* DSM 19835^T lack complete Pentose phosphate pathway; only *Eudoraea adriatica* DSM 19308^T and

Arenibacter troitsensis DSM 19835^T has a complete D-Galacturonate degradation; only *Eudoraea adriatica* DSM 19308^T and *Muriicola jejuensis* EM44^T has complete Glycogen biosynthesis, for Nucleotide sugar biosynthesis, only *Maribacter polysiphoniae* KCTC 22021^T has a complete pathway; *Zeaxanthinibacter enoshimensis* TD-ZE3^T, *Robiginitalea biformata* HTCC2501^T, *Muriicola jejuensis* EM44^T and *Poritiphilus flavus* R33^T has a complete Propanoyl-CoA metabolism; only *Eudoraea adriatica* DSM 19308^T and *Cellulophaga tyrosinoxydans* EM41^T has complete Formaldehyde assimilation; For Amino acid metabolism, Valine biosynthesis, Leucine biosynthesis and Lysine biosynthesis are found in all these strains; *Maribacter polysiphoniae* KCTC 22021^T lacks a complete Lysine biosynthesis; Strain D16^T, *Maribacter aquivivus* DSM 16478^T and *Arenibacter troitsensis* DSM 19835^T has complete Methionine degradation; D16^T lacks complete Methionine degradation, *Zeaxanthinibacter enoshimensis* TD-ZE3^T, *Robiginitalea biformata* HTCC2501^T, *Cellulophaga tyrosinoxydans* EM41^T and *Arenibacter troitsensis* DSM 19835^T has complete Proline biosynthesis, only *Maribacter polysiphoniae* KCTC 22021^T lacks Histidine biosynthesis/degradation. Complete metabolism pathways which were only found in the strain D16 were TCA cycle, second carbon oxidation, Methionine degradation, Isoleucine biosynthesis, NAD biosynthesis and *Hem*e biosynthesis.

Secondary Metabolite Biosynthesis Clusters Analysis

Clusters related to secondary metabolite biosynthesis were identified using the antiSMASH program and found differences in gene cluster abundance and diversity between strain D16^T and close relative genera (Supplementary Figure 11**)**. Common gene clusters encoding of strain D16^T, *Eudoraea adriatica* DSM 19308^T, *Robiginitalea biformata* HTCC2501^T, *Muriicola jejuensis* EM44^T and *Zeaxanthinibacter enoshimensis* TD-ZE3^T were T3PKS: Chal_sti_synt_C, T3PKS: Chal_sti_synt_N, terpene: Lycopene_cycl, terpene: phytoene_synt, 2OG-FeII_Oxy, adh_short_C2, Alpha-amylase, PP-binding, RimK, t2fas, short-chain dehydrogenase/reductase SDR, aminotransferase class-III, oxidoreductase, Polyprenyl synthetase, BC transporter ATP-binding protein, TetR family transcriptional regulator, ND family efflux transporter MFP subunit and dehydrogenase. Gene clusters found in strain D16^T were arylpolyene: APE_KS2, 8-amino-7-oxononanoate synthase, GATase_7, Aminotran_3, Peptidase_M16_C, PF04055 and PF06968. Based ResFinder builds upon assembly and BLAS (Clausen et al. 2016), the tigecycline-resistance tet(X) gene was found in the genome of the strain D16^T. Strain D16^T was susceptible to tetracycline, Which was consistent with that *Flavobacteriaceae* is a potential ancestral source of tigecycline resistance gene tet (X) (Zhang et al. 2020).

Carbohydrate-Active Enzymes (CAZymes)

Studies have shown that polysaccharides utilization was found in many *Bacteroidota* bacteria of marine(Francis et al. 2019). Genomes of D16^T and closely related genera were analyzed by the CAZy database. Strain D16^T, *Eudoraea adriatica* DSM 19308^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T, *Robiginitalea biformata* HTCC 2501^T, *Muriicola jejuensis* EM44^T, *Maribacter vaceletii* DSM 25230^T and *Cellulophaga tyrosinoxydans* EM41^T contain 160, 129, 109, 112, 87, 231 and 237 carbohydrate-active enzymes, respectively (Figure 4). carbohydrate-active enzymes including 46 GHs were found in strain D16^T, which was less than *Eudoraea adriatica* DSM 19308^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T and *Robiginitalea biformata* HTCC 2501^T, *Muriicola jejuensis* EM44^T and *Cellulophaga tyrosinoxydans* EM41^T. The experimental results show strain D16^T, *Eudoraea adriatica* DSM 19308^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T and *Robiginitalea biformata* HTCC 2501^T, *Muriicola jejuensis* EM44^T and *Cellulophaga tyrosinoxydans* EM41^T can utilize various carbon sources (Table1), which was consistent with the genome annotation results.

Table 3. Differential characteristics of strain D16^T (*Aoguangibacterium sediminis* gen. nov., sp. nov.) and members of related genera of the family *Flavobacteriaceae.*

Strains: 1, D16^T(data from this study); 2, *Eudoraea adriatica* DSM 19308^T(Alain et al. 2008); 3, *Muriicola jejuensis* EM44^T(Kahng et al. 2010); 4, *Zeaxanthinibacter enoshimensis* TD-ZE3^T(Asker et al. 2007); 5, *Robiginitalea biformata* HTCC2501^T(JC and SJ 2004); 6, *Maribacter arcticus* KOPRI 20941^T (Cho et al. 2008); 7, *Cellulophaga tyrosinoxydans* EM41^T (Kahng et al. 2009); 8, *Arenibacter troitsensis* DSM 19835^T(Nedashkovskaya et al. 2003); 9, *Poritiphilus flavus* R33^T(Wang et al. 2020).

+, Positive; 2, negative; ND, not determined; W, weak

Characteristic	1	2	3	4	5	6	7	8	9
Pigmentation*	R/O	-	Y/O	Y	Y/O	-	-	-	+
Flexirubin	+	-	-	-	-	N	+	-	-
Gliding motility	-	-	-	+	-	+	+	-	-
Reaction to oxygen	A	A	A	A	A	A	A	A	A
Growth at 42 °C	+	-	-	-	+	-	-	-	-
Nitrate reduction	-	-	+	-	-	+	+	-	+
Oxidase/catalase production	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
β-galactosidase	-	+	+	-	-	-	+	+	+
Glucose acidification	-	-	+	+	-	-	+	-	+
Degradation of:									
Starch	+	-	-	+	+	-	+	+	+
Aesculin	-	+	+	+	+	-	N		N
Gelatin	-	-	-	+	-	-	+	+	+
DNA	-	N	N	-	-	N	-	N	N
Cellulose	+	-	-	-	-	N	-	-	N
Urea	+	-	-	-	-	-	-	-	+
Arginine	-	-	N	N	-	N		-	+
16S rRNA gene sequence similarity (%)#	100	93	91.9	92.3	92.3	91.9	91.5	90.9	92.5

*Y, Yellow; O, orange; DO, dark orange.

O, Obligate aerobe; A, facultative anaerobe.

#Similarities calculated in reference to the 16S rRNA gene sequence of strain D16^T

Table 4. Phenotypic and genotypic characteristics of strain D16^T (*Aoguangibacterium sediminis* gen. nov., sp. nov.)

Characteristic	Strain D16^T
Temperature range for growth (°C) (optimum)	10-42 (30-33)
NaCl range for growth (%, w/v) (optimum)	0-5 (1)
pH range for growth (optimum)	5.5-9.5 (7.0)
Bioch*emi*cal properties (API 20E)	
o-nitrophenyl-*β*-D-galactopyranoside, Urease	+
arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase	-
citrate utilization, H_2S production, tryptophan deaminase, indole production	
Voges–Proskauer reaction, gelatinase, glucose, mannitol, nositol, sorbitol, rhamnol	
sucrose, melibiose, amygdalin, arabinose	
Enzymic activities (API ZYM)	
alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase	+
trypsin, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase,	
β-glucosidase, N-acetyl-*β*-glucosaminidase	
lipase (C14), cystine arylamidase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase,	-
α-glucosidase, *α*-mannosidase, *α*-fucosidase	
Substrate assimilation	
1% Sodium Lactate, Fusidic Acid, D-Serine, Troleandomycin, Rifamycin SV ,Minocycline, Lincomycin, Glucuronamide, Nalidixic Acid, Potassium Tellurite, Acetoacetic Acid, Sodium Bromate	+
Vancomycin, L-Malic Acid, Bromo-Succinic Acid, Lithium Chloride	
Acetic Acid, Formic Acid, Aztreonam, Sodium Butyrate	
Tween 20, Tween 40, Tween 60, Tween 80	-
Degration of macromolecules	
Amylase, Casein	+
Cellulase, Agar, Algin, Casein, Dnase, starch, urea	-
Susceptibility to antibiotics	
Tetracycline, ampicillin, rifampin, carbenicillin, vancomycin.	+
Polar lipids	DPG，PE，AGL，GL，AL，PGS
Quinones	MK-6
DNA G+C content (mol%)	42.8

Phenotypic and Bioch*emi*cal Characteristics

Strain D16^T formed aerobic, non-motile, red, mesophilic and slightly halotolerant colonies with diameters of 1-3 mm after growing for 96 hours on modified MA at 33 °C. The cell was Gram-stain-negative, motile by gliding and spherical (0.2-0.4 μm wide and 1.3-2.0 μm long) (Figure 2). Strain D16^T could adapt to growth at higher temperatures (Table 3). The strain D16^T was able to hydrolysis cellulose, urea and starch but was negative for degradation of arginine, Tweens (80, 60, 40, and 20), DNA, gelatin, aesculin agar, alginate and casein. Strain D16^T produced Poly-*β*-hydroxybutyrate, similar to the related strains. Strain D16^T was susceptible to tetracycline, ampicillin, rifampin, carbenicillin, vancomycin. The results are summarized in Table 4. Comparative analysis of physiological and bioch*emi*cal strain D16^T and related strains were summarized in Supplementary Figure 12.

C*hem*otaxonomic Analysis

Major components of fatty acids (>10 %) of strain D16^T were iso-$C_{15:0}$ (32.0 %) and $C_{16:0}$ (10.5 %) , iso-$C_{15:0}$ was also detected as the major fatty acids in type species of all type genus in *Flavobacteriaceae*. Listed in Table 5 and iso-$C_{15:1}$ was also the major component in type strains of *Eudoraea adriatica* DSM 19308^T, *Muriicola jejuensis* EM44^T, *Robiginitalea biformata* HTCC 2501^T, *Maribacter arcticus* KOPRI 20941^T and *Arenibacter antarcticus* R18H21^T. The five most closely phylogenetic relatives. However, $C_{16:0}$, one of the major fatty acids in strain D16^T, was detected only in trace amounts in type strains of *Muriicola jejuensis* EM44^T, *Robiginitalea biformata* HTCC 2501^T and *Arenibacter antarcticus* R18H21^T (Table 5). Polar lipids of strain D16^T included Diphosphatidylglycerol (DPG)，Phosphatidylethanolamine (PE)，Aminoglycolipid (AGL)，glycolipid (GL)，Aminolipid (AL) and one unidentified lipid PGS

(Supplementary Figure 13). The respiratory quinone of strain D16^T was menaquinone-6 (MK-6), typical features of the family *Flavobacteriaceae* (J.-F. Bernardet et al., 2002).

Environmental Distribution

The 16S rRNA sequence of the strain D16^T was added to the SILVA SSU database ((Quast et al. 2013); SSU Ref NR release 138.1) and aligned by the ARB version 7.0 software package (Ludwig et al. 2004). The phylogenetic position of strain D16^T and relative genera were determined by comparative analyses of these sequences in the ARB version 7.0 software package. Compare 16S rRNA sequence the strain D16^T, those sequences of strains whose sequence similarity between D16^T was higher 95% was selected from SILVA SSU 138.1. The environmental distribution of these strains and relative genera was analyzed by the isolation source form environmental sequences affiliated with relative genera in the European Nucleotide Archive (ENA)[8]. Supplementary Table S1 lists all the 16S rRNA gene sequences used.

The difference between the proposed *Aoguangibacterium* genus and other related genera was also reflected in different environmental isolation source. The majority of representatives of the genus *Muriicola*, *Eudoeaea*, *Maribacter*, *Cellulophaga* and *Zeaxanthinibacter* inhabit marine environments, soil or fresh water and are expected to have a metabolism adapted to aerobic or microaerophilic conditions. as shown in Supplementary Figure 14, the proposed *Aoguangibacterium* genus of the family *Flavobacteriaceae* mainly was isolated from hypoxic or microhypoxic environments, like marine sediments or hypersaline sediments. Only a few representatives were found in aerobic environments such as sea water or freshwater. Strain D16^T, isolated from marine sediments, is similar to isolation source of the proposed *Aoguangibacterium* genus of the family *Flavobacteriaceae,* which was consistent with results of anaerobic experiments and denitrification pathway annotated in genome of D16^T. Specific analysis of the isolation source of members of the proposed *Aoguangibacterium* genus and related genera were presented in Supplementary Table S1.

Discussion

The majority of representatives in the family *Flavobacteriaceae* were distributed in the marine environment and are expected to adapt to micro-aerobic and anaerobic environments. One of the family *Flavobacteriaceae* was isolated from human blood (Leyer et al. 2020). *Candidatus Endobryopsis*, one of the family *Flavobacteriaceae,* can live in symbiosis with the alga Bryopsis sp (Zan et al. 2019). Marine sediments, especially offshore sediments, which was a habitat where posses extremely active material transformation and energy flow. The offshore area, where the water depth is less than 50M no more than 2% of the total ocean area, transported to the ocean almost 48% of the global inflows of organic carbon fluxes into the oceans (Arndt et al. 2013).

In the genome of strain D16^T, 1373 genes were predicted, including 1340 protein-coding genes，which include genes associated with CO2 fixation and denitrification, which can be inferred that the strain D16^T was also autotrophic. Some differences in key metabolic pathways also supported the separate evolution of the strain D16^T and other related genera of the family *Flavobacteriaceae*. (succinate dehydrogenase/fumarate reductase (*sdh*C/ *frd*C) catalyzes the generation of Fumarate and Succinate in the Citrate cycle (TCA cycle, Krebs cycle) and functions as a key switch in the regulation of carbon flux distribution (Akram 2014). Both the substrates and products of *sdh*C/ *frd*C are involved in the tricarboxylic acid cycle, anaplerosis and energy anabolism. *sdh*C/ *frd*C was present in the genomes of the proposed genus D16^T. However, it was missing in the genome of other relative genera.

Furthermore, it is involved in the second carbon oxidation of the Citrate cycle, which was also only present in D16^T. Only strain D16^T has a complete tricarboxylic acid (TCA) cycle, which is related to the metabolism of the three major substances and is also the hub of energy metabolism (Steffens et al. 2021). In addition, for the *heme* biosynthesis pathway, uroporphyrinogen-III synthase (*hem*D, UROS) was present in D16^T, which was missing in other relative genera. The cyclic tetrapyrrole *heme* is used as a prosthetic group in a wide variety of different proteins in almost all organisms. It is often essential for vital bio*chem*ical processes such as aerobic and anaerobic respiration and photosynthesis (Layer 2021), which is consistent with the that the D16^T has CAM (Crassulacean acid) metabolism) metabolic pathways. This indicates it can perform photosynthesis (Yurkov and Beatty 1998; Béjà et al. 2002; Karl 2002; Oz et al. 2005; Fuchs et al. 2007; Suzuki and Béjà 2007).

The evolutionary divergence among members the strain D16^T and other relative genera was also reflected by different growth performances under aerobic conditions. The strain D16^T cannot grow under microaerobic/aerobic conditions. However, other relative genera can also grow under microaerobic/aerobic conditions, relative to the environmental distribution of members of the proposed genus *Aoguangibacterium* and other relative genera. (JC and SJ 2004; Asker et al. 2007; Alain et al. 2008; Hu et al. 2015; Wang et al. 2020).

As a new genus of the family *Flavobacteriaceae*, strain D16^T has many features that differentiate it from other bacterial groups of *Flavobacteriaceae*. Such as cellulose degradation and flexirubin biosynthesis, absent in other related genera, was found in the strain D16^T. Compared to the high levels of iso-$C_{15:0}$ and 3-OH $i_{17:0}$ of most members of *Flavobacteriaceae,* the strain D16^T only has a low proportion of iso-$C_{15:0}$ and 3-OH $i_{17:0.}$ Most members can degrade lipid (Tweens20 40, 60, and 80). However, the strain D16^T can not degrade Tweens 20, 40, 60, and 80. Many family *Flavobacteriaceae* were positive oxidase production, but oxidase was absent in the strain D16^T.

Taxonomic Conclusion

In this study, a novel strain D16^T with carbon fixation and denitrification was isolated from marine sediment from China. Phylogenetic trees based on genomes and 16S rRNA genes illustrated that the strain D16^T formed a separate branch with related genera (*Eudoraea*, *Poritiphilus, Zeaxanthinibacter*, *Robiginitalea* and *Muriicola*) and was distinctly different from other genera of family *Flavobacteriaceae* (Figures 1–2). Compare strain D16^T with closest relative genera; the ANI, POCP, DDH and AAI values didn't exceed the recommended critical point for genus or species division (Table 5), revealing that the

strain D16^T represents a novel genus. When comparing the strains D16^T and close relatives, Their physiological and bioc*hem*ical characteristics were markedly different. Therefore, strain D16^T indicated a new genus in the family *Flavobacteriaceae*.

Description of *Aoguangibacterium sediminis* gen. nov., sp. nov.

Description of *Aoguangibacterium* gen. nov.

Aoguangibacterium (Ao.guang.i.bac.te'ri.um. N.L. masc. n. bacterium a rod; N.L. neut. n. Aoguangibacterium, a rod named after Ao Guang, Dragon King of the Eastern Sea in Chinese folklore).

Gram-stain-negative, non-motile, Aerobic, non-spore-forming, red, mesophilic, **d**enitrifying, carbon-fixing and slightly halotolerant. Pigments are Red or Orange. Oxidase-negative and catalase-positive. Neutrophilic. Polar lipids include (DPG□AGL□ GL □ AL □PGS). The predominant quinone is MK-6. Major cellular fatty acids were (iso-$C_{15:0}$, 32.0 %) and ($C_{16:0}$, 10.5 %). The G+C content of the DNA is 42.8 mol %. Phylogenetically, the genus *Aoguangibacterium* belongs to the family *Flavobacteriaceae*, phylum *Bacteroidota*, showing a distant relatedness to the marine genera *Eudoraea, Zeaxanthinibacter, Robiginitale, Cellulophaga, Muriicola* and *Poritiphilus*. The type species is D16^T.

Aoguangibacterium sediminis sp. nov.

Aoguangibacterium sediminis (se.di'mi.nis. L. gen. neut. n. *sediminis*, of sediment).

Displays the following characteristics in addition to those given in the genus description. Cells are 0.2-0.4 μm in width and 1.3-2.0 μm in length; On MA, colonies (1.0-3.0 mm in diameter) are round, red, smooth, non-motile and shiny. Growth occurs at 10-42 °C (optimum,30-33 °C), at pH 5.5-10.0 (optimum,7.0) and at 0–5% (optimum, 1.0%) NaCl; Starch, cellulose and urea are hydrolyzed, but aesculin, gelatin, DNA and arginine are not. O-nitrophenyl-*β*-D-galactopyranoside and Urease are produced. arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, tryptophan deaminase, indole production, Voges–Proskauer reaction, gelatinase, glucose, mannitol, inositol, sorbitol, rhamnol, sucrose, melibiose, amygdalin and arabinose are not produced. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-glucosidase, N-acetyl-*β*-glucosaminidase activities are positive. lipase (C14), cystine arylamidase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *α*-mannosidase, *α*-fucosidase activities are negative. Acid is produced from 1 % Sodium Lactate, Fusidic Acid, D-Serine, Troleandomycin, Rifamycin SV, Minocycline, Lincomycin, Glucuronamide, Vancomycin, L-Malic Acid, Bromo-Succinic Acid, Nalidixic Acid, Lithium Chloride, Potassium Tellurite, Acetoacetic Acid, Acetic Acid, Formic Acid, Aztreonam, Sodium Butyrate, Sodium Bromate. Predominant cellular fatty acids (representing 10 % of the total fatty acids) are (iso-$C_{15:0}$, 32.0 %) and ($C_{16:0}$, 10.5 %). The DNA G+C content of the type strain is 42.8 mol %.

The type strain, D16^T (=MCCC 1H00463^T= KCTC 82746^T), was isolated from coastal sediment from Xiaoshi Island, Weihai, China. The DNA G + C content of the type strain is 42.8%. Accession numbers are MZ695036 (16S rRNA gene) and JAJTJC000000000 (whole genome).

Abbreviations

DPG, Diphosphatidylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; HPLC, High-performance liquid chromatography; CHES 2-(Cyclohexylamino) ethanesulfonic acid; MES2-(N-Morpholino) ethanesulfonic acid; MOPS 3-(N-Morphine) propanesulffsonic acid; TAPS N-Tris,(hydroxymethy)methyl-3-aminopropanesulfonic acid; KCTC, Korean Collection for Type Cultures; MIDI, Microbial Identification System; POCP, Percentage of conserved proteins.

Declarations

Footnotes

- www.bacterio.net
- https://www.ezbiocloud.net/identify
- https://blast.ncbi.nlm.nih.gov/Blast.cgi
- http://soap.genomics.org.cn/soapdenovo.html
- https://itol.embl.de/
- https://github.com/2015qyliang/POCP
- https://ggdc.dsmz.de/ggdc.php
- https://www.ebi.ac.uk/ena/browser/home
- http://ekhidna2.biocenter.helsinki.fi/AAI/

Author Contributions Z-LY isolated the Strain D16^T. Z-LY, KW, Y-WJ, Y-HD, Z-JD and D-SM performed material preparation, experimental operation, data collection, and analysis. Z-LY and D-SM wrote the manuscript. Z-JD and D-SM performed project guidance and critical revision of manuscripts. All authors contributed to the article and approved the submitted version.

Funding This work was supported by the National Natural Science Foundation of China (41876166, 32070002, and 31900091), the National Science and Technology Fundamental Resources Investigation Program of China (2019FY100700), and the China Postdoctoral Science Foundation (2019M652369).

Conflict of interest This article does not contain any studies with human participants or animals performed by any of the authors. Moreover, all authors read and approved the final manuscript. All the authors declare that they have no direct or indirect conflict of interest.

Ethical Approval It is the original work of the author. The work described has not been submitted elsewhere for publication, in whole or in part, and all authors listed carry out the data analysis and manu-script writing.

Acknowledgements

Scanning electron microscopy was supported by Physical-C*hem*ical Materials Analytical & Testing Center of Shandong University at Weihai. New specie name thanks advice and help of Professor Aharon Oren.

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Tables

Tables 5 is not available with this version.

Figures

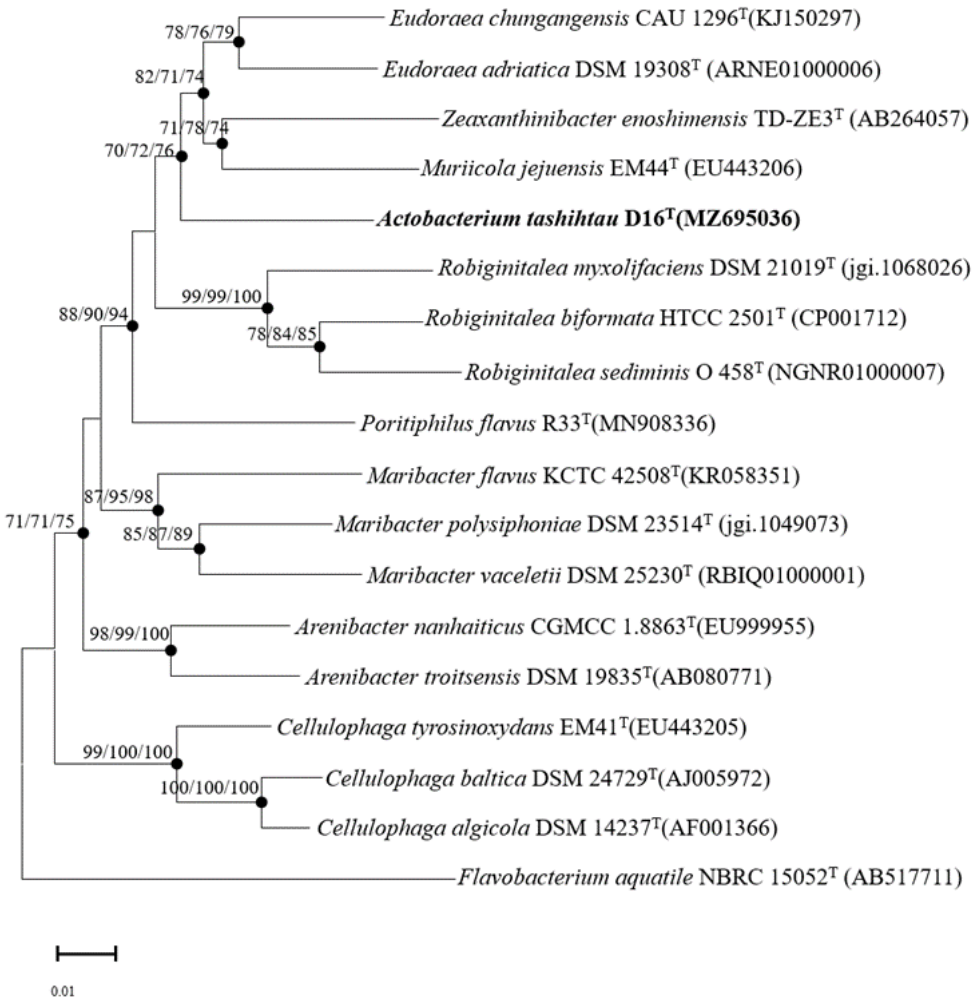

Figure 1

Phylogenetic tree constructed based on 16S rRNA gene sequences of strain D16^T and its closely related species of the family *Flavobacteriaceae*. The tree was constructed using the neighbor-joining method (NJ), maximum-parsimony method (MP) and maximum-likelihood method (ML). Bootstrapping was carried out with 1,000 replicates. The numbers at the branch node values are estimated from NJ and ML, respectively. The node values below 70% are not shown. The black circles at the node represent the congruent topology between NJ and ML. *Flavobacterium aquatile* NBRC 15052^T was selected as the outgroup. Scale bars: 0.01 represented the nucleotide substitution per position

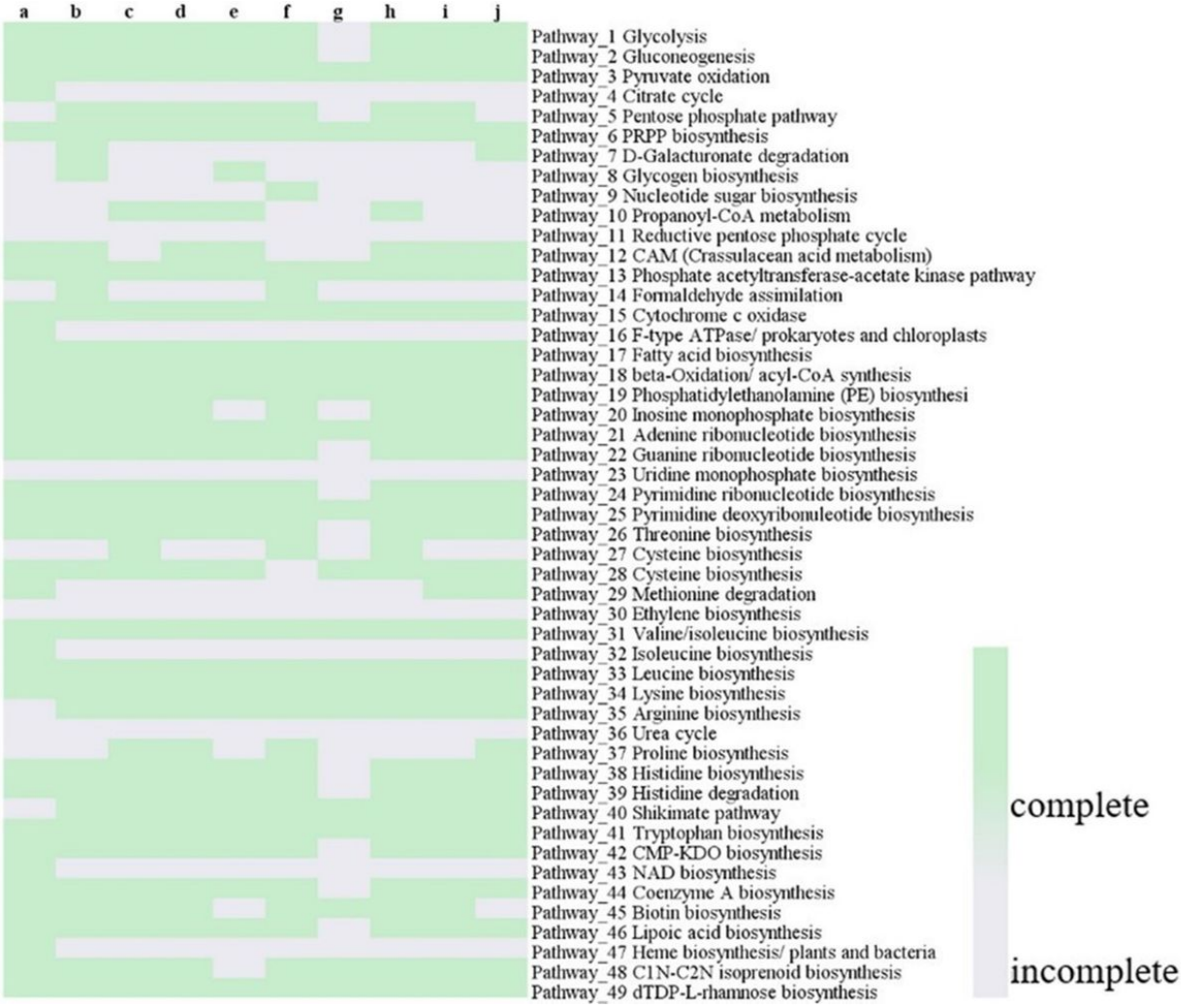

Figure 2

Heatmaps of comparative genomic analysis between strain D16^{T} and related strains. heatmap clustered with 49 pathways according to the annotation of the KEGG. a, Strain D16^{T}; b, *Eudoraea adriatica* DSM 19308^{T}; c, *Zeaxanthinibacter enoshimensis* TD-ZE3^{T}; d, *Robiginitalea biformata* HTCC2501^{T}; e, *Muriicola jejuensis* EM44^{T}; f, *Cellulophaga tyrosinoxydans* EM41^{T}; g, *Maribacter polysiphoniae* KCTC 22021^{T}; h, *Poritiphilus flavus* R33^{T}; i, *Maribacter aquivivus* DSM 16478^{T}; j, *Arenibacter troitsensis* DSM 19835^{T}.

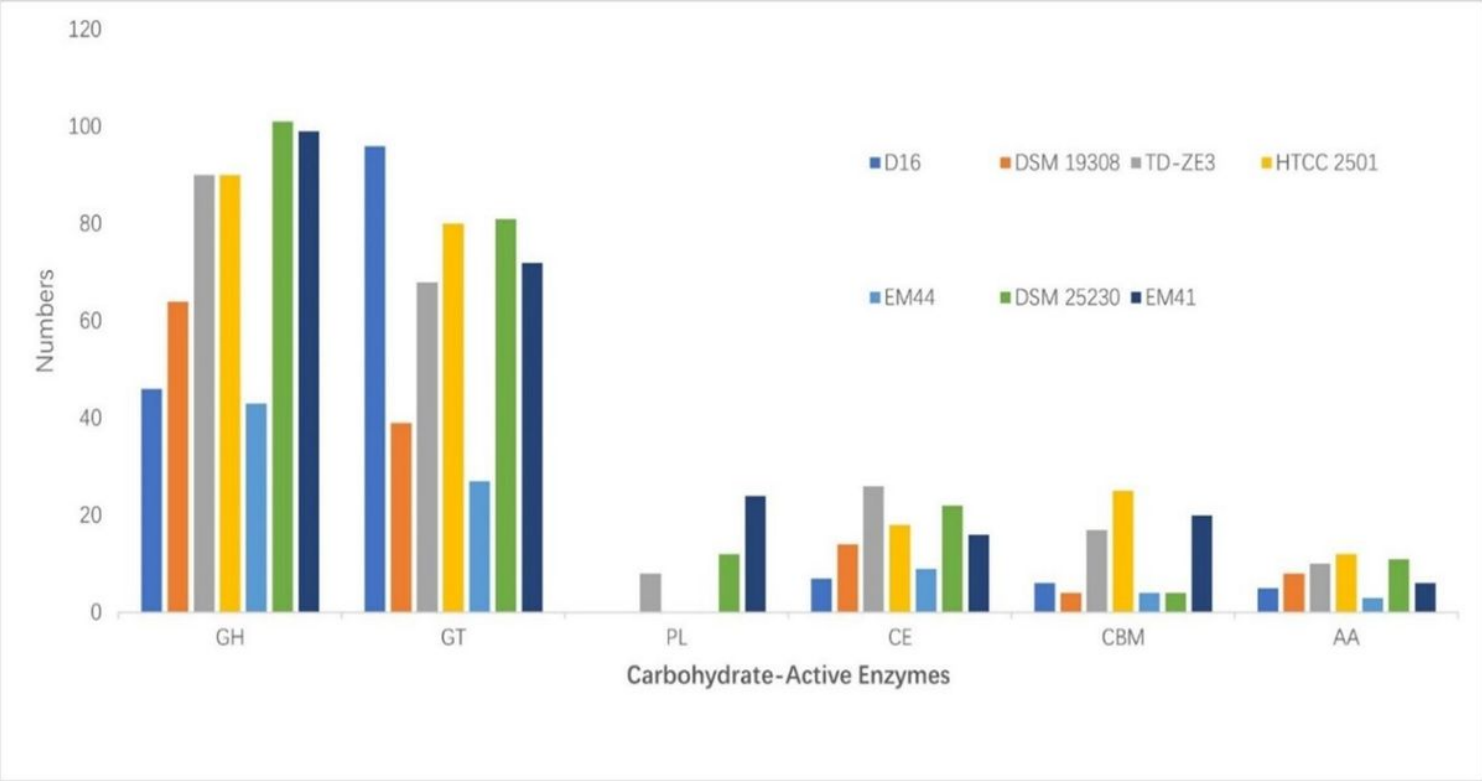

Figure 3

Histogram of predicted carbohydrate-active enzymes (CAZymes) in strain D16^T, *Eudoraea adriatica* DSM 19308^T, *Zeaxanthinibacter enoshimensis* TD-ZE3^T, *Robiginitalea biformata* HTCC 2501^T, *Muriicola jejuensis* EM44^T, *Maribacter vaceletii* DSM 25230^T and *Cellulophaga tyrosinoxydans* EM41^T. Glycoside Hydrolase (GH)、Glycosyltransferase (GT)、Polysaccharide Lyase (PL)、Carbohydrate Esterase (CE)、Carbohydrate Binding Domain (CBM)、Accessory Enzyme （AA）.

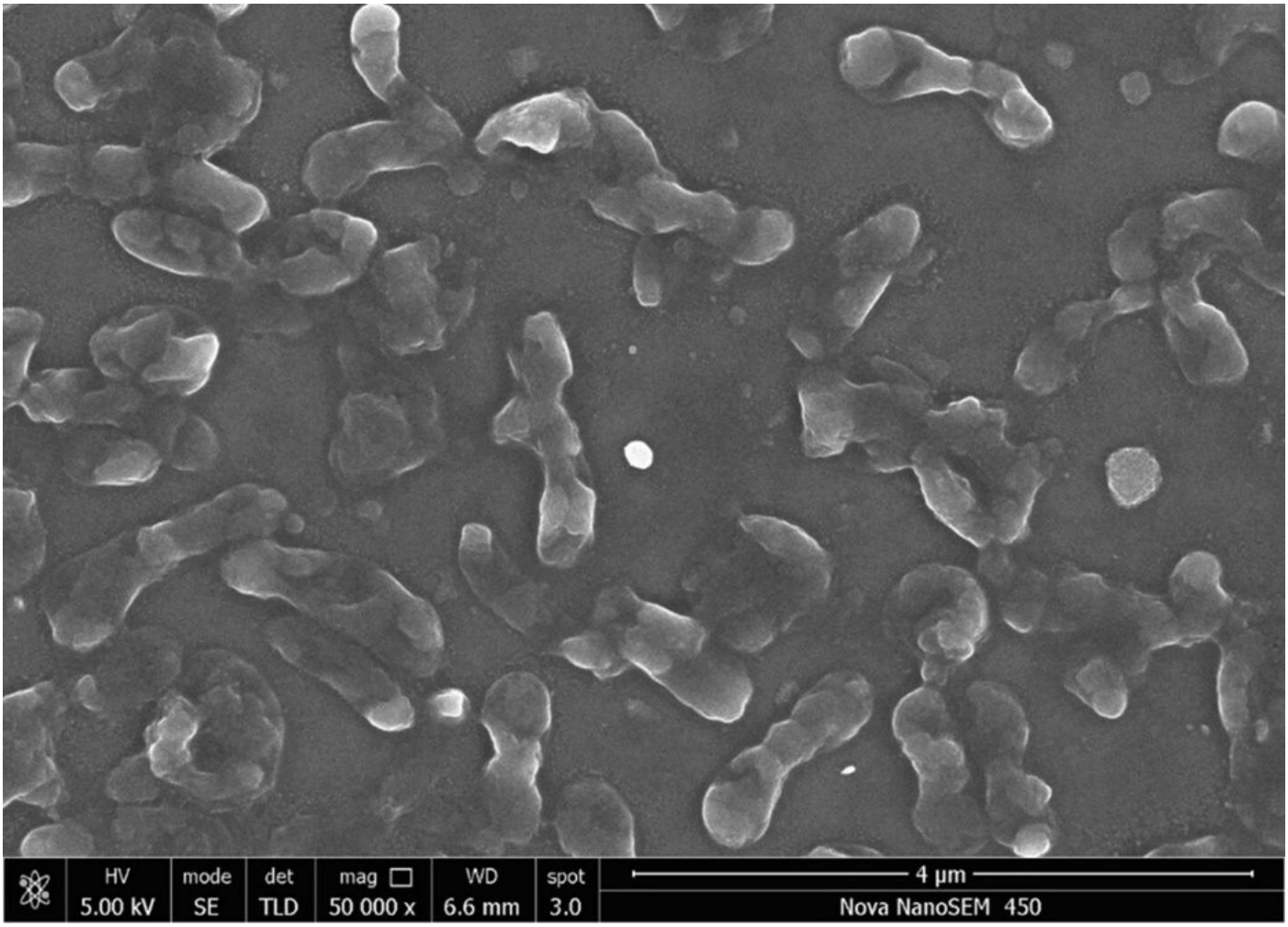

Figure 4

Scanning Electron Microscope image of strain D16^{T} cultivated at 30 °C for three days on 2216E agar medium. Bar, 4 µm.

Supplementary Files

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- SupplementaryTableS1ofD16.xlsx
- SupplementarymaterialsofD16.pdf