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ORIGINAL PAPER

Sulfitobacter alexandrii sp. nov., a new microalgae growth-promoting bacterium with exopolysaccharides bioflocculanting potential isolated from marine phycosphere

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Abstract Marine phycosphere harbors unique cross-kingdom associations with enormous ecological significance in aquatic ecosystems as well as relevance for algal biotechnology industry. During our investigating the microbial composition and bioactivity of marine phycosphere microbiota (PM), a novel lightly yellowish and versatile bacterium designated strain AM1-D1^T was isolated from cultivable PM of marine dinoflagellate *Alexandrium minutum* amtk4 that produces high levels of paralytic shellfish poisoning toxins (PSTs). Strain AM1-D1^T demonstrates notable bioflocculanting bioactivity with bacterial exopolysaccharides (EPS), and microalgae growth-promoting (MGP) potential toward its algal host. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain AM1-D1^T was affiliated to the members of genus *Sulfitobacter* within the family *Rhodobacteraceae,* showing the highest sequence similarity of 97.9% with *Sulfitobacter noctilucae* NB-68^T, and below 97.8% with other type strains. The complete genome of strain AM1-D1^T consisted of a circular 3.84-Mb chromosome and five circular plasmids (185, 95, 15, 205 and 348 Kb, respectively) with the G+C content of 64.6%. Low values obtained by phylogenomic calculations on the average nucleotide identity (ANI, 77.2%), average amino acid identity (AAI, 74.7%) and digital DNA-DNA hybridization (dDDH, 18.6%) unequivocally separated strain AM1-D1^T from its closest relative. The main polar lipids were identified as phosphatidylglycerol, phosphatidylethanolamine,

The DDBJ/EMBL/GenBank accession numbers for 16S rRNA gene sequences of the strain AM1-D1T is MH197128. The complete genome sequences of strain AM1-D1T have been deposited at DDBJ/EMBL/GenBank under the accession numbers CP018076 to CP018081 for its circular chromosome and five circular plasmids, respectively.

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phosphatidylcholine, diphosphatidylglycerol, one unidentified phospholipid and one unidentified lipid. The predominant fatty acids (> 10%) were $C_{18:1}$ ω7c, $C_{19:0}$ cyclo ω8c and $C_{16:0}$. The respiratory quinone was Q-10. The genome of strain AM1-D1^T was predicted to encode series of gene clusters responsible for sulfur oxidation (*sox*) and utilization of dissolved organic sulfur exometabolites from marine dinoflagellates, taurine (*tau)* and dimethylsulfoniopropionate (DMSP) (*dmd*), as well as supplementary vitamin B_{12} (*cob*), photosynthesis carotenoids (*crt*) which are pivotal components during algae-bacteria interactions. Based on the evidences by the polyphasic characterizations, strain AM1-D1^T represents a novel species of the genus *Sulfitobacter*, for which the name *Sulfitobacter alexandrii* sp. nov. is proposed. The type strain is AM1-D1^T (= CCTCC 2017277T = KCTC 62491^T).

Keywords Algae-bacteria interactions · Phycosphere microbiota · *Sulfitobacter alexandrii* · *Alexandrium minutum* · Microalgae growth-promoting bacterium · Paralytic shellfish poisoning toxins

Abbreviations

AAI	Average amino acid identity
ABI	Algae-bacteria interactions
ANI	Average nucleotide identity
MGPB	Microalgae growth-promoting bacteria
dDDH	Digital DNA–DNA hybridization
DPG	Diphosphatidylglycerol
DMSP	Dimethylsulfoniopropionate
EPS	Exopolysaccharides
HAB	Harmful algal blooms
MA	Marine agar
MB	Marine broth
ML	Maximum likelihood
MP	Maximum parsimony
NJ	Neighbour joining
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PHB	Polyhydroxybutyrate
PL	Phospholipids
PM	Phycosphere microbiota
PMP	Phycosphere microbiome project
UBCG	Up-to-date bacterial core gene

Introduction

The genus *Sulfitobacter* was established by Sorokin (1995), and belongs to *Roseobacter* group which is the most widely distributed bacteria in the marine environment, and harbors enormous ecological significance during oceanic elemental cycles and interactions with marine eukaryotes (Buchan 2005; Wagner-Döbler and Biebl 2006). *Sulfitobacter pontiacus* isolated from the Black Sea is the type species of this genus (Sorokin 1995). At the time of writing, the genus *Sulfitobacter* includes 23 validated species (https://lpsn.dsmz.de/genus/ sulfitobacter) that were all isolated from marine environments (Wang et al 2021). Marine phycosphere harbors dynamic and cross-kingdom algae-bacteria interactions (ABI) which play crucial roles in aquatic ecosystems (Seymour et al. 2017). Within the phycosphere interface, the exopolysaccharides (EPS) produced by algae-associated bacterial consortia serves as one essential intermediary component during those dynamic interactions (Amin et al. 2012; Cho et al. 2004; Yang et al. 2021; Zhang et al. 2020). Previously, we performed the Phycosphere Microbiome Project (PMP) to convey microbial structures of phycosphere microbiota (PM) of marine harmful algal blooms (HAB) dinoflagellates (Duan et al. 2020; Yang et al. 2018, 2020a, b; Yang et al. 2020a, b, c; Zhang et al. 2015a, b, 2020; Zhou et al. 2021). We found *Sulfitobacter* spp. was one dominant genus distributed in diverse PM of six toxic HAB *Alexandrium* spp. (Fig. S1) (Zhang et al. 2015a, b). After isolating the cultivable PM, a new lightly yellowish and versatile bacterium designated strain AM1-D1^T was isolated from *Alexandrium minutum* amtk4 that produces high levels of paralytic shellfish poisoning toxins (PSTs) (Chou et al. 2004; Zhang et al. 2015b; Yang et al. 2020a, b, c). We found that strain AM1-D1^T produces active bioflocculanting EPS (Duan et al. 2020; Mu et al. 2019), and also demonstrates obvious microalgae growth-promoting (MGP) ability. To specify its phylogenetic position, in this study, we proposed that strain AM1-D1^T represents a novel species of the genus *Sulfitobacter* based on polyphasic characterizations. Additionally, whole-genomic mining of strain AM1-D1^T gained insight into the application of this new isolate for furthering exploration of bacterial PST biosynthesis and detailed

mechanisms underlying those dynamic algae-bacteria interactions (Amin et al. 2012; Seymour et al. 2017).

Materials and methods

Bacterial strains and culture conditions

Strain AM1-D1^T was isolated from the cell culture of *Alexandrium minutum* amtk4 according to our protocols described previously (Yang et al. 2018a; Zhang et al. 2015a, 2020; Zhou et al. 2021). The isolation medium of marine agar (MA, Difco, BD) 2216 was supplemented with algal culture extract of LZT09 at approximate 0.05 mg/L (Yang et al. 2021). The strain was routinely cultivated on MA at 30 °C, or maintained as a glycerol suspension (20%, v/v) and stored at − 80 °C for long-term preservation. Five reference type strains, including *S. noctilucae* NB-68^T (= KCTC 32122^T = JCM 18833^T = DSM 100978^T), *S. noctilucicola* NB-77^T (= KCTC 32123^T = JCM 18834^T) and *S. geojensis* MM-124^T (= KCTC 32124^T = JCM 18835^T) purchased from Korean Collection for Type Cultures (KCTC, Republic of Korea), *Roseobacter denitrificans* OCh 114^T (= ATCC 33942^T = DSM 7001^T = JCM 21267^T) obtained from the Japan Collection of Microorganisms (JCM, Japan), and *S. pacificus* SCM2-10^T (= LMG 27113^T = NBRC 109915^T) obtained from the NITE Biological Resource Center (NBRC) were used for the comparative study of the taxonomic position of strain AM1-D1^T.

16S rRNA phylogenetic analysis

PCR amplification of bacterial 16S rRNA gene was performed using an universal bacterial primers of 27F/1492R according the standard procedure (Yang et al. 2021; Zhang et al. 2021), and sequenced at MajorBio (Shanghai, China). The identifications of phylogenetic neighbor and the calculations of gene similarity values using the 16S rRNA gene sequences obtained by both PCR amplification, and extracted 16S rRNA region from the bacterial genomes were achieved using online EzTaxon server (http://www.ezbiocloud.net). Reference sequences of type strains were downloaded from the NCBI database (www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using MEGA 7.0 after multiple alignment of the data via ClustalW. Phylogenetic distances were calculated according to neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods. In each case, bootstrap values were calculated based on 1000 replications to evaluate the phylogenetic tree topology.

Phenotypic, physiological and biochemical characterizations

Cell morphology was observed by light microscope (Olympus CX21) and transmission electron microscopy (TEM) JEM-1200; JEOL, Tokyo, Japan). The morphology of cell colonies were observed after 24 h incubation on MA plates at 30 °C. Motility was tested microscopically under the phase-contrast mode by the hanging drop technique. Growth range and optimum at different temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C), and pH values (4.0–11.0, at 0.5 unit interval) was investigated in marine broth (MB) incubated at 30 °C for 2 days. Growth at various NaCl concentrations (0–11.0%, w/v, interval of 0.5%) was investigated in MB at 30 °C for 2 days. The following sterile buffer system: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH_2PO_4/0.1 M NaOH; pH 9.0–10.0, 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11.0, 0.05 M Na_2HPO_4/0.1 M NaOH, was used to adjust the desired pH of the test media. Growth under anaerobic condition was determined after incubation in an anaerobic chamber (Bactron EZ-2; Shellab) grown on MA plate and cultivated at 30 °C for 2 week. Growth was determined by monitoring OD_{600nm} using an Agilent spectrophotometer. Oxidase activity was determined using a 1% (v/v) solution of tetramethyl-*p*-phenylenediamine. Catalase activity was detected by assessing production of bubbles after addition of a drop of 3% (w/v) H_2O_2. Hydrolysis of aesculin, casein, DNA, gelatin, starch and Tween 80, Voges–Proskauer and methyl red tests, production of indole, phenylalanine deaminase, phosphatase, nitrate and nitrite reduction and Simmon's citrate were determined as described by Smibert and Krieg (1994). Activities of extracellular enzymes were determined by using API 20E, API 20NE and API ZYM strips (bioMérieux, Marcyl'E´toile, France) according to the manufacturer's instructions.

Chemotaxonomic characteristics

Strain AM1-D1^T was incubated in MB in order to extract the fatty acids. The exponential-phase of the cell culture was harvested, and the fatty acids were analyzed as described by Sasser (1990). Identification and quantification of cellular fatty acids were performed using the Sherlock Microbial Identification System (MIDI) (Sherlock version 6.1; MIDI database TSBA6.0). Polar lipids of were extracted using a chloroform/methanol system, separated by two-dimensional silica gel TLC, and analyzed according to the procedures described by Tindall et al. (1987). Respiratory quinones were extracted and determined by HPLC method as described previously (Hiraishi et al. 1996).

Phylogenomic calculations and functional genes analysis

The genome sequences of type strains of *Sulfitobacter* were available until December 2020, and downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genome). The up-to-date bacterial core gene set (UBCG) was used to construct a phylogenetic tree using the genomes of strain AM1-D1^T and other type strains of *Sulfitobacter*. DNA G+C content of strain AM1-D1^T was calculated based on its whole-genome sequence. Three measures of similarities based on the average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA-DNA hybridization (dDDH) values were calculated using online OrthoANI tool and GGDC tool (http://ggdc.dsmz.de/distcalc2.php), respectively, using the default parameters. Additionally, the identification of essential functional genes and clusters related to quorum sensing (*lux*), dimethylsulfoniopropionate (DMSP) (*dmd),* photosynthetic capacity (*puf*, *bch*), carbon monoxide oxidation (*cox*), *tau* gene, sulfur oxidation (*sox*), Vitamin B_{12} (*cob*), carotenoids (*crt*), and nitrogen metabolism (*nap*A, nasA, narB, narG, norB, nirS) in the genomes of strain AM1-D1^T and its close relatives were performed using BLAST based on InterPro (https://www.ebi.ac.uk/interpro) database and IMG database (https://img.jgi.doe.gov).

Core-genome alignment and phylogenetic analysis

All predicted protein-coding genes and amino acids annotated from available 16 genome of type strains of the genus *Sulfitobacter* were selected and compared using an all-versus-all BLAST search. This analysis identified shared reciprocal best matches (defined as $> 70\%$ nucleotide identity or $> 40\%$ amino acid identity) in all pair-wise genome comparisons (core orthologues, CO) of 17 type strains of *Sulfitobacter* including strain AM1-D1^T. The core orthologous genes were individually aligned using MUSCLE (Tatusov et al. 2000). The resulting nucleotide alignments were concatenated to create a core-genome alignment using IQ-TREE v1.6.12 (http://www.iqtree.org). The phylogenetic tree was reconstructed by neighbour-joining method with MEGA 7 (https://www.megasoftware.net/home). Additionally, the core orthologous and unique proteins were used to construct a Venn diagram using the VennPainter tool (Lin et al. 2016).

Bacterial EPS bioflocculanting and MGP bioactivity assays

Extraction of bacterial EPS produced by strain AM1-D1^T and bioflocculating activity evaluation were performed according to our procedures reported previously (Duan et al. 2020; Mu et al 2019; Zhang et al 2021). The prepared EPS was dissolved in distilled water for furthering bioflocculation activity (BFC) assay (Mu et al. 2019). Briefly, the measures using kaolin clay suspension flocculation (KCSF) assay calculated as flocculation rate (FR) were used and performed at a 96-well microplate mode with at least triplicates. Microalgae growth-promoting (MGP) activity of strain AM1-D1^T toward *A. minutum* amtk4 in a co-culture system was performed as reported previously (Gonzalez and Bashan 2000).

Results and discussion

Morphological, physiological and biochemical characteristics

Strain AM1-D1^T was observed to form lightly yellowish colonies when grown on MA at 30 °C for 2 days. Cells of strain AM1-D1^T were Gram-negative,

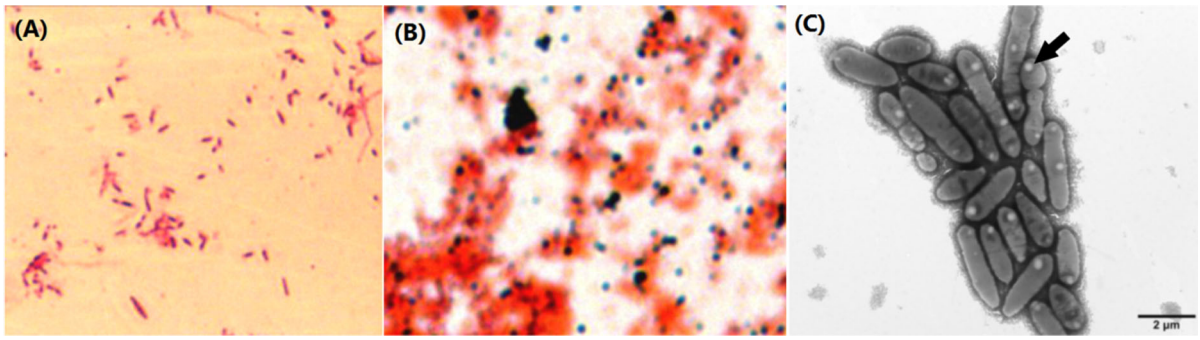


Fig. 1 Morphological images of strain AM1-D1^T cells grown in MB medium at 30 °C for 2 days, observed by optical microscopy after Gram-staining (**a**), and Sudan Black staining showing the PHA granules (**b**), and transmission electron microscopy with black arrow indicating the PHA granules inside (**c**)

rod-shaped with polyhydroxyalkanoate (PHA) granules inside (Fig. 1). It's motile, aerobic and weak positive for anaerobic growth. TEM observation showed that the cells of strain AM1-D1^T were approximately 0.3–0.4 μm wide and 0.7–2.1 μm long (Fig. S1), which were smaller than the related type strains. Growth occurred at 15–37 °C (optimum, 30 °C) at pH 5–10 (optimum, pH 7.0) in the presence of 1–4% (w/v) NaCl (optimum, 3%). Oxidase and catalase activities were both positive. Other phenotypic features distinguishing strain AM1-D1^T from its close relatives are summarized in the species description (Table 1). Compared with its five close relatives, strain AM1-D1^T exhibited a wider temperature grow range, but a narrower NaCl grow range. In API 20NE tests, only two strains, *S. pacificus* SCM2-10^T and *S. geojensis* MM-124^T were positive for nitrate reduction, but strain AM1-D1^T and other three type strains were all negative for nitrate reduction. In API ZYM tests, strain AM1-D1^T and *S. pacificus* SCM2-10^T were positive for acid phosphatase, naphthol-AS-BI-phosphohydrolase, but positive for lipase (C14).

It's attractively noted that for the bacterial isolation sources, total 8 type strains of genus *Sulfitobacter,* including AM1-D1^T, *S. delicatus* and *S. dubius* (Ivanova et al. 2004), *S. faviae* (Kumari et al. 2016), *S. pacificus* (Fukui et al. 2015), *S. porphyrae* (Fukui et al. 2014), *S. pseudonitzschiae* (Hong et al. 2015), *S. undariae* (Park et al. 2015) and *S. algicola* (Wang et al 2021) were isolated from diverse marine dinoflagellates and planktons. This interesting finding may indicate those species might involve in potential cross-kingdom associations with their hosts (Amin et al. 2012).

16S rRNA phylogenetic analysis

The extracted 16S rRNA gene sequences of strains AM1-D1^T and its close relatives were subjected to phylogenetic analysis. The similarity identification using EzBioCloud alignment indicated that strain AM1-D1^T shared 16S rRNA gene similarities of 97.9% with *S. noctilucae* NB-68^T, and less than 97.8% with all other *Sulfitobacter* species, and below 97.5% with all type strains of the genus *Roseobacter* (Shiba 1991). Phylogenetic tree constructed based on NJ method demonstrated that AM1-D1^T form a monophyletic group compared with other species of the genus *Sulfitobacter*, which was outside the group formed by the type strains of *S. noctilucae*, *S. noctilucicola*, *S. pacificus*, *S. geojensis* and *S. sabulilitoris* (Fig. 2). The phylogenetic relationship was also supported by the ML (Fig. S2) and MP trees (Fig. S3).

Phylogenomic comparison of Sulfitobacter species genomes

The phylogenomic comparisons based on ANI, AAI and dDDH values between strain AM1-D1^T and other type strains of the genus *Sulfitobacter* with available genomes are showed in Fig. S4. The ANI, AAI and dDDH values between strain AM1-D1^T and *S. noctilucae* NB-68^T were 77.2, 74.7 and 18.6%, respectively. For *S. sabulilitoris* HSMS-29^T, the ANI, AAI and dDDH values were 78.0, 71.2 and 19.3%, respectively. For *Roseobacter denitrificans* OCh 114^T, those three values were 71.8, 68.4 and 14.2%, respectively. All values are far lower than the thresholds (95–96% for ANI, 97% for AAI and 70%

Table 1 Differential characteristics of strain AM1-D1^T and its phylogenetically close relatives

Isolation source	1 Marine phycosphere	2 Coastal seawater	3 Coastal seawater	4 Marine red alga	5 Coastal seawater	6 Marine sediment
Flagellum	−	−	−	+	+	+
PHA	+	+	+	+	+	−
Pigments	Lightly yellowish	Lightly yellowish	Lightly yellowish	Cream	Lightly yellowish	Pink
Cell size (μm)						
Width	0.3–0.4	0.6–0.9	0.4–0.8	0.6–0.8	0.5–0.9	0.6–0.9
Length	0.7–2.1	1.5–3.1	1.0–2.6	1.5–2.5	1.2–2.3	1.0–2.0
Growth						
Temperature range (°C, optimum)	15–37 (30)	4–30 (25)	4–30 (25)	5–30 (25)	4–30 (25)	4–30 (25)
pH range (optimum)	5–10 (7)	5–9 (7)	5–10 (7)	5–9 (7)	5.5–9 (7–8)	7.5–9.5 (8)
NaCl range (%, w/v, optimum)	1–4 (3)	1–6 (3)	1–6 (3)	1–6 (3)	1–6 (3)	1–6 (3)
API ZYM tests						
Alkaline phosphatase	+	−	+	+	−	+
Lipase (C14)	−	+	+	−	+	+
Leucine arylamidase	+	−	−	+	−	+
Cystine arylamidase	+	+	+	−	+	+
Trypsin	−	+	+	−	+	+
Acid phosphatase	+	−	−	+	−	+
Naphthol-AS-BI-phosphohydrolase	+	−	−	+	−	−
α-galactosidase	−	+	+	−	+	+
API 20NE tests						
Nitrate reduction	−	−	−	+	+	+
Urease	−	−	+	−	−	+
DNA G+C content (mol%)	64.6	58.3 [b]	57.1 [b]	56.5 [c]	57.8 [b]	59.0 [d]
Polar lipids profile	DPG, PG, PE, PC [a]	DPG, PG, PE [a]	DPG, PG, PE, PC [b]	PG, PE, PC [c]	PG, PE, PC [b]	PG, PE, PC [d]

Strains: 1, *S. alexandrii* AM1-D1^T; 2, *S. noctilucae* NB-68^T; 3, *S. noctilucicola* NB-77^T; 4, *S. pacificus* SCM2-10^T; 5, *S. geojensis* MM-124^T; 6, *Roseobacter denitrificans* OCh 114^T. All strains are Gram-stain-negative, aerobic and rod-shaped, and require Na^+ for growth. In API 20NE tests, all strains were negative for indole production, arginine dihydrolase. In API ZYM tests, all strains were positive for esterase (C4), esterase lipase (C8), valine arylamidase, but negative for α-chymotrypsin, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. + : positive, − : negative

Data from the original study, [a]this study, [b]Kwak et al. (2014), [c]Fukui et al. (2015), [d]Shiba et al. (1991)

for dDDH) generally accepted for new species delineation (Meier-Kolthoff et al. 2013; Richter and Rosselló-Móra 2009). Additionally, based on the constructed phylogenomic tree (Fig. 3), strain AM1-D1^T formed a separate branch clustered with *S. sabulilitoris* HSMS-29^T which was isolated from a marine sand sample collected from the Yellow Sea, South Korea (Park et al. 2019). In addition, the comparison of genomic characteristics including the genomic sizes, G+C contents and gene numbers also can clearly distinguish strain AM1-D1^T from its closely related species within the genus *Sulfitobacter,* and also those phylogenetic neighbors within other genus. These results strongly support the species delineation of strain AM1-D1^T from other type strains

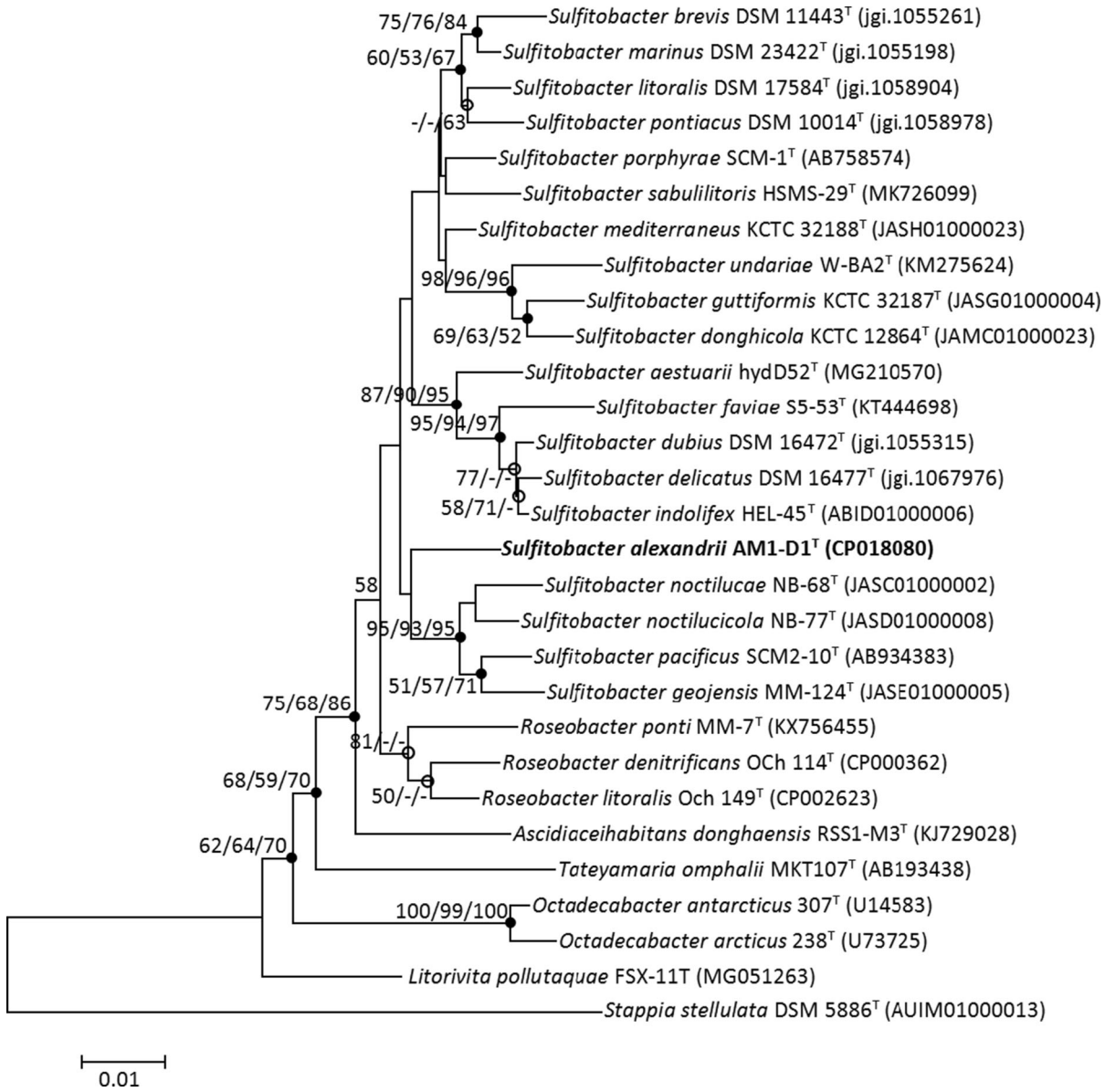


Fig. 2 Neighbor-joining (NJ) phylogenetic tree of strain AM1-D1^T and type strains of the genus *Sulfitobacter* and related taxa within the family Rhoae based on 16S rRNA gene sequences. Bootstrap values (> 50%) are expressed as percentages of 1000 replicates. *Stappia stellulata* DSM 5886^T was used as an outgroup. *Bar*, 0.01 substitutions per nucleotide position

of the genus *Sulfitobacter*, and hence strain AM1-D1^T is proposed as a novel species of *Sulfitobacter*.

Chemotaxonomic analysis

As shown in Table 2, the chemotaxonomic analysis of the cellular fatty acids showed that the predominant fatty acid profiles (> 10%) of strain AM1-D1^T were $C_{18:1}$ ω7c (42.1%), $C_{19:0}$ cyclo ω8c (23.5%) and $C_{16:0}$ (15.2%). Additionally, two other fatty acid compositions with lower portions including $C_{18:1}$ ω7c 11-methyl (7.3%) and $C_{18:0}$ (3.7%) were also found in strain AM1-D1^T. The result was in agreement with those of the other members of *Sulfitobacter*. However, strain AM1-D1^T also possessed high amount of $C_{19:0\ cyclo}$ ω8c (23.5%) and minor amount of $C_{12:1}$

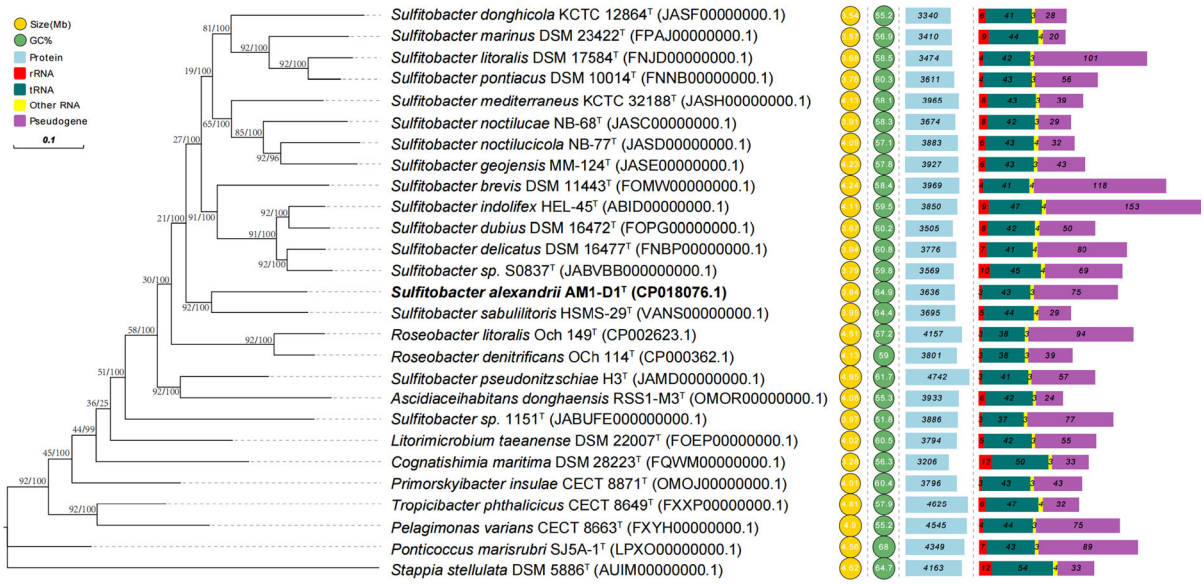


Fig. 3 Phylogenetic tree of strain AM1-D1^T and other type strains of the genus *Sulfitobacter* constructed by up-to-date bacterial core gene (UBCG) set, and comparison of the genomic characteristics including genomic size, DNA G+C content (%), numbers of predicted protein-coding genes, rRNA, tRNA and pseudogenes. *Stappia stellulata* DSM 5886^T is selected as an outgroup. Gene support index (the number of individual gene trees presented the same node in total genes used) (left) and bootstrap values (right) are given at nodes. *Bar*, 0.1 nt substitution rate (K_{nuc}) units

Table 2 Cellular fatty acid compositions of strain AM1-D1^T and five reference strains of the genus *Sulfitobacter*

Fatty acids	1	2	3	4	5	6
$C_{10:0}$ 3-OH	*tr*	3.3	4.5	2.8	3.4	3.1
$C_{16:0}$	15.2	4.5	2.7	3.3	4.1	2.3
$C_{18:0}$	3.7	1.2	2.2	1.5	1.7	3.3
$C_{19:0}$ cyclo $\omega 8c$	23.5	–	–	–	–	–
$C_{12:1}$ 3-OH	2.7	–	–	–	–	–
$C_{18:1}$ $\omega 7c$	42.1	85.7	87.1	76.7	81.7	86.7
$C_{18:1}$ $\omega 7c$ 11-methyl	7.3	3.2	2.7	8.0	5.5	2.9
*Summed feature**						
3	1.9	*tr*	–	–	–	*tr*

Strains: 1, *S. alexandrii* AM1-D1^T; 2, *S. noctilucae* NB-68^T; 3, *S. noctilucicola* NB-77^T; 4, *S. pacificus* SCM2-10^T; 5, *S. geojensis* MM-124^T; 6, *Roseobacter denitrificans* OCh 114^T. All data were obtained from this study. Values represent percentage of total fatty acids contents. –, not detected; *tr*, trace amounts (< 1%)

*Summed feature 3 contains $C_{16:1}$ $\omega 7c$ and/ or $C_{16:1}$ $\omega 6c$

3-OH (2.7%). These unique components can be used to clearly distinguish strain AM1-D1^T from its closely related species. The isoprenoid quinone of strain AM1-D1^T was ubiquinone-10 (Q-10). The main polar lipids of strain AM1-D1^T were determined as diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), one unidentified phospholipid (PL) and one unidentified lipid (L). Unlike its closet relative, *Sulfitobacter noctilucae* NB-68^T had DPG, PG, PE and minor amounts of three unidentified lipids (L1-3) as the major polar lipids profile (Fig. S5). Both characterizations were consistent with the common chemotaxonomic profile of the other members of *Sulfitobacter* (Sorokin 1995; Fukui et al. 2014, 2015; Park et al. 2015, 2019; Wang et al 2021).

Genome comparison of strain AM1-D1^T

Circular representation of the genome of strain AM1-D1^T including one circular chromosome and five circular plasmids is shown in Fig. 4. The size of the whole genome of strain AM1-D1^T is 4.69 Mb with one chromosome of 3.84 Mb with a DNA G+C content of 64.9%. And the lengths of five plasmids were 185, 95, 15, 205 and 348 Kb with their corresponding G+C contents of 65.6, 60.3, 58.7, 63.7 and 61.6%, respectively (Table 1). Based on the complete genome sequence, the whole genomic DNA G+C

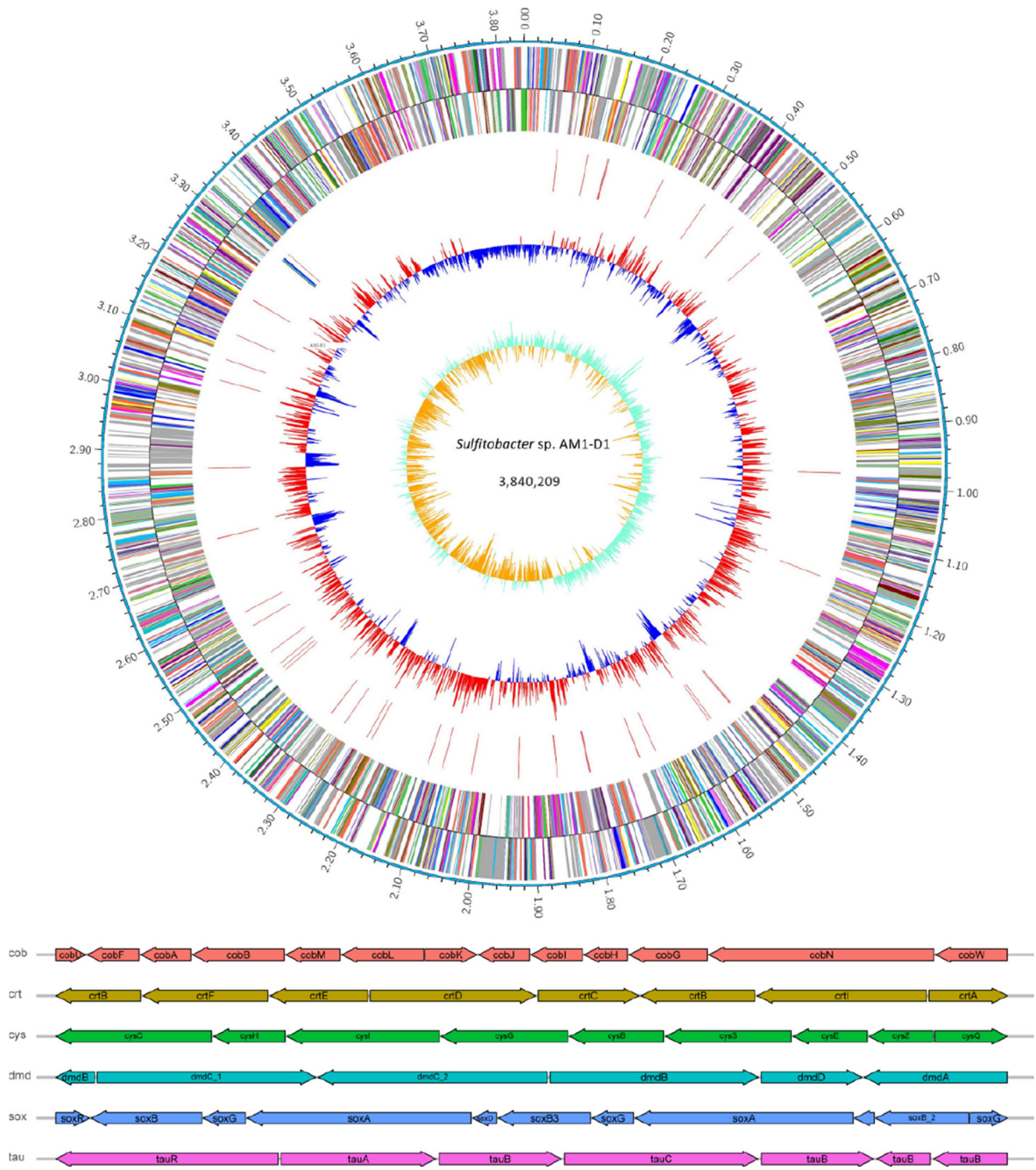
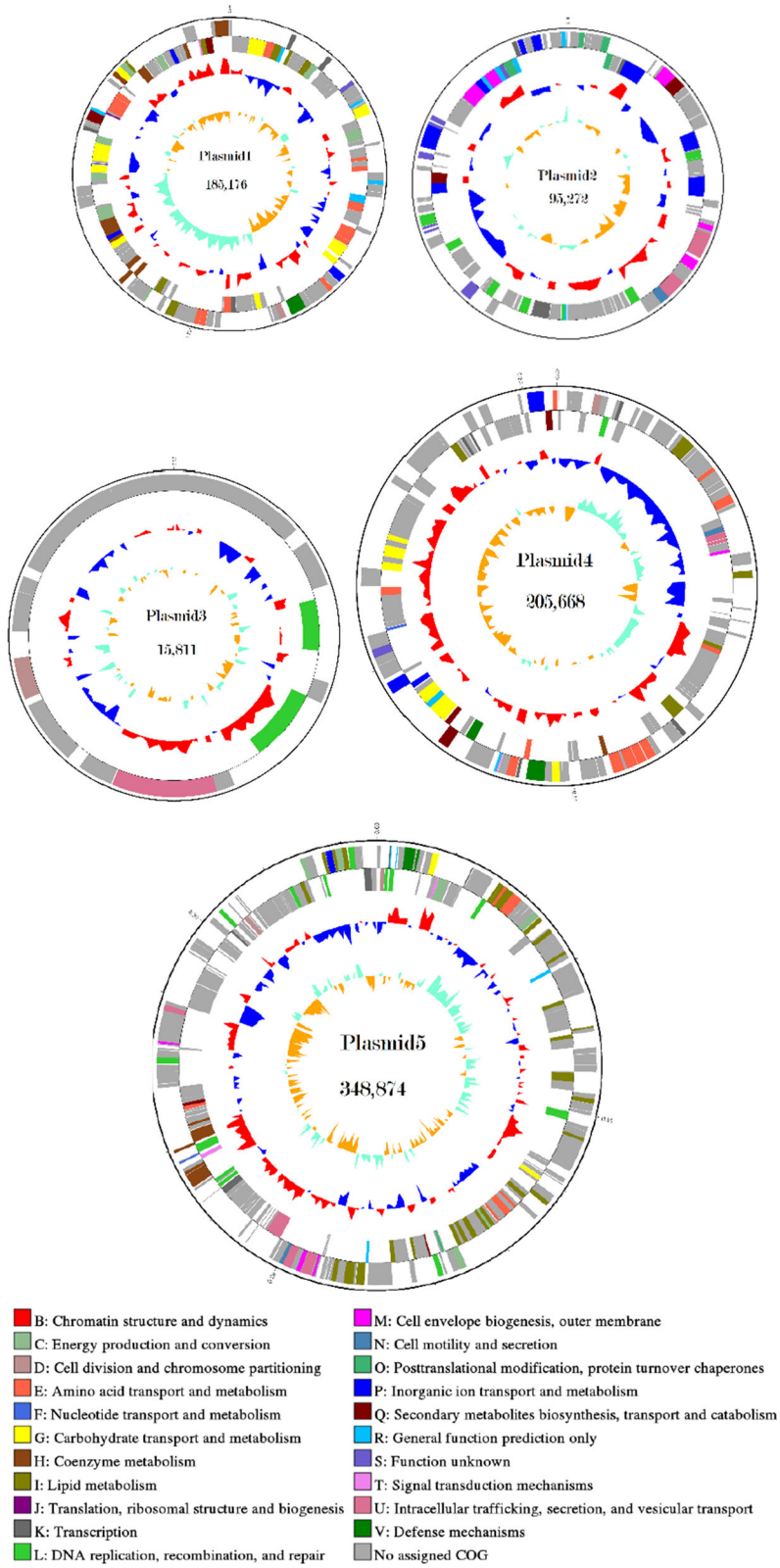


Fig. 4 Circular representation of the genome of strain AM1-D1^{T} including one circular chromosome and five circular plasmids. Typical bacterial metabolic gene clusters (*cob, crt, cys, dmd, sox* and *tau*) found in the genome were also shown. The scale of the genome size was shown in the outer line. From the outer to inner circle: the two outer circles show the predicted protein-coding sequences (CDs) on the plus and minus strand. Different colors in these two circles show genes with different COG categories; the third circle shows rRNA and tRNA (red); the fourth and inner fifth circles show G+C content and G+C skew, respectively

Fig. 4 continued



content of strain AM1-D1^T was calculated as 64.6%, which was higher than its five closely related type strains. Transmission electron microscopy showed that the cells of strain AM1-D1^T were approximately 0.3–0.4 μm wide and 0.7–2.1 μm long (Fig. S1), which were smaller than the related strains.

According to NCBI database records, total 17 genomes of type strains of *Sulfitobacter* were chosen to conduct the core- and pan-genome analysis. The constructed two functional curves showed that the total gene families and core gene families reached to maximal and minimal values of 16,295 and 1530, respectively (Fig. 5). The gene accumulation curve exhibited an increasing tendency with the increasing number of the pan-genome genes. But the core-genome curve showed a descending trend (Fig. 5). Therefore, the pan-genome was considered in an open state since the pan-genome trend curve did not reach a plateau with the increasing numbers of bacterial strains. In contrast, the core genome curve was thought to be conserved. As shown in Fig. 5, the gene occurrence plot showed that 1530 core genes were shared by 17 type *Sulfitobacter* genomes, accounting for 33.2% to 45.8% of the genome repertoire, respectively. Additionally, about 7.7% to 25.5% of unique genes were only found in *S. dubius* DSM 16472^T and *S. pseudonitzschiae* DSM 26824^T, respectively, indicating a species-specific profile within *Sulfitobacter* members.

Comparative analysis of function genes

The distribution of functional categories of core and unique genes was compared and the result is shown in Fig. 6. It indicated that the most abundant functions in core genes of *Sulfitobacter* spp. were associated with amino acid transport and metabolism, general function prediction only, translation, ribosomal structure and biogenesis, and energy production and conversion, which were closely related to necessary nutrients obtaining from varied environments and maintaining a basic lifestyle. Function unknown accounted for a large proportion, which indicated current lacking of in-depth revealing of *Sulfitobacter* genomes. For KEGG assignment, the five most abundant core genes were distributed into KEGG categories about amino acid metabolism, carbohydrate metabolism, energy metabolism, nucleotide metabolism and overview, suggesting adaptive evolution exist within member of the genus *Sulfitobacter*. However, genes related to cellular, development, digestive system, immune diseases, immune system and substance dependence were only assigned in core genes. Functional analysis revealed that the abundances and categories of core and unique genes assigned into KEGG categories were diverse in member of the genus *Sulfitobacter*. Additionally, series of essential functional gene clusters responsible for vitamin B_{12} (*cob*), photosynthesis pigments carotenoids (*crt*), cysteine biosynthesis (*cys*), sulfur oxidation (*sox*), and utilization of two dissolved organic sulfur (DOS) exometabolites from marine dinoflagellates, taurine (*tau)* and DMSP (*dmd*) (Landa et al. 2019; Landry et al. 2018), were found in the genome of strain AM1-D1^T (Fig. 3), which are pivotal components governing the dynamic algae-bacteria interactions (Amin et al. 2012).

Bacterial EPS bioflocculanting and MGP potential

Based on our bacterial bioflocculanting assay, the extracted EPS produced by strain AM1-D1^T were subjected to BFC bioactivity screening assay (Mu et al. 2019). The comparison of BFC of series of EPS concentrations on KCSF was performed. Pane A in Fig. 7 shows the concentration-dependent manner of BBF bioactivity of EPS produced by strain AM1-D1^T. It can be seen that the BFC reached the maximum of 92.5 ± 6.2% at 0.40 g L^{-1} of bacterial EPS produced by strain AM1-D1^T, which exhibiting higher bioflocculanting deficiency compared with the strains we previously reported (Duan et al. 2020; Yang et al. 2020a, b, c; Yang et al. 2021; Zhang et al 2021). For MGP assay, strain AM1-D1^T demonstrated obvious microalgae growth-promoting activity when co-

cultured with host *A. minutum* amtk4 (pane B in Fig. 7). Those results clearly indicate that strain AM1-D1^T is a new MGPB isolated from marine phycosphere, and produces novel bioactive bioflocculating EPS with potential environmental and biotechnological implications (Mu et al 2019; Duan et al. 2020; Yang et al 2021; Zhang et al 2021).

Taxonomic conclusion

On the basis of phenotypic, phylogenetic and genomic analyses, strain AM1-D1^T represents a novel species of the genus *Sulfitobacter*. Therefore, we formally propose *Sulfitobacter alexandrii* sp. nov., and AM1-D1^T as the type strain. Strain AM1-D1^T appears to be a versatile bacterium with potential for environmental and biotechnological applications including microalgae growth-promoting potential and the production of natural EPS biosurfactans.

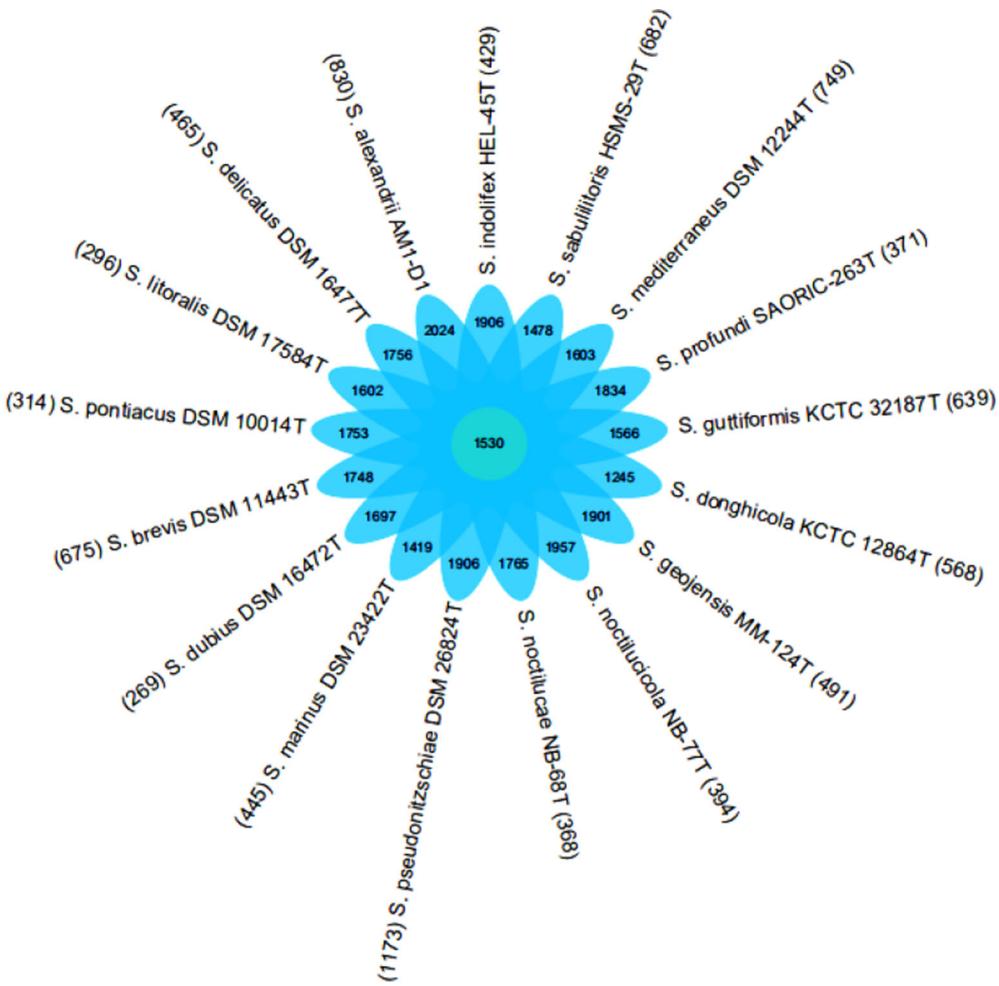
Description of Sulfitobacter alexandrii sp. nov.

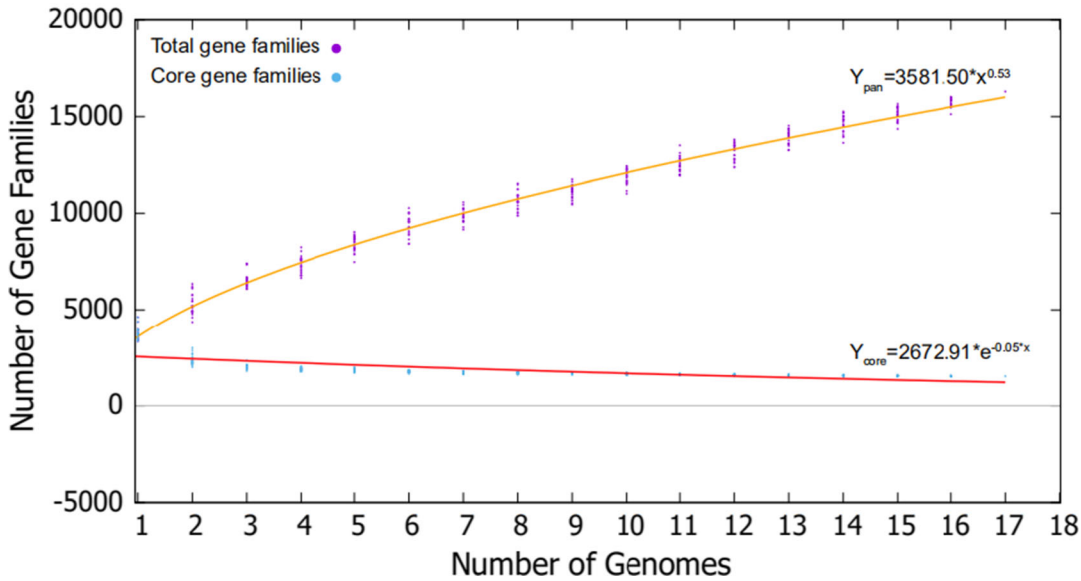
Sulfitobacter alexandrii (a.le.xan'dri.i. N.L. gen. n. *alexandrii* of the dinoflagellate *Alexandrium minutum*, the isolation source of the type strain).

Cells are Gram-stain-negative, aerobic, non-sporulating, non-motile, rod-shaped with approximately 0.3–0.4 μm wide and 0.7–2.1 μm long with PHA granules inside. Colonies are circular, convex, smooth, lightly yellowish grown on MA for 2 days at 30 °C. Growth occurs at 15–37 °C, optimum at 30 °C. Grows at 1.0–4.0% (w/v) NaCl with an optimum at 3.0% (w/v) NaCl. Tolerates a range of pH between 5.0 and 10.0 with the optimum at 7.0. Aesculin and Tween 20 are hydrolysed. Gelatin, Tween 40 and 80 are not hydrolysed. Nitrate is reduced to nitrite. Indole is not produced from tryptophan and glucose fermentation does not occur. Oxidase and catalase are both positive. Positive for urease activities, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, and negative for lipase (C14), cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The predominant cellular fatty acids are $C_{18:1}$ ω7c, $C_{19:0}$ cyclo ω8c and $C_{16:0}$. The predominant isoprenoid quinone is Q-10. The main polar lipids are phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The genome has 3.84-Mb circular chromosome and five circular plasmids of the lengths of 185, 95, 15, 205 and 348 Kb, respectively, with the genomic DNA G+C content of 64.6 mol%.

The type strain is AM1-D1^T (= CCTCC AB 201696^T = KCTC 52626^T), which was isolated from phycosphere microbiota of highly-toxic and HAB dinoflagellate *Alexandrium minutum* amtk4 which was collected from a Milkfish culture pond at TungKang Taiwan during an algal bloom occurred in July, 1994, and then routinely cultivated in the laboratory. The DDBJ/EMBL/GenBank accession numbers for 16S rRNA gene sequences of the strain AM1-D1^T is MH197128. The complete genome sequences of strain AM1-D1^T have been deposited at DDBJ/EMBL/GenBank under the accession numbers CP018076, CP018077, CP018078, CP018079, CP018080 and CP018081 for its circular chromosome and five circular plasmids, respectively.

Fig. 5 Venn diagram of core- and pan-genome analysis of members of *Sulfitobacter* and the constructed function curves ▶


(830) S. alexandrii AM1-D1
S. indolifex HEL-45T (429)
S. sabulilitoris HSMS-29T (682)
(465) S. delicatus DSM 16477T
S. mediterraneus DSM 12244T (749)
(296) S. litoralis DSM 17584T
S. profundi SAORIC-263T (371)
(314) S. pontiacus DSM 10014T
S. guttiformis KCTC 32187T (639)
(675) S. brevis DSM 11443T
S. donghicola KCTC 12864T (568)
(269) S. dubius DSM 16472T
S. geojensis MM-124T (491)
(445) S. marinus DSM 23422T
S. noctilucicola NB-77T (394)
(1173) S. pseudonitzschiae DSM 26824T
S. noctilucae NB-68T (368)
2024
1906
1478
1756
1603
1602
1834
1753
1566
1530
1748
1245
1697
1901
1419
1906
1765
1957


Total gene families
Core gene families
Y_pan=3581.50*x^0.53
Y_core=2672.91*e^-0.05*x
Number of Gene Families
Number of Genomes
20000
15000
10000
5000
0
-5000
1
2
3
4
5
6
7
8
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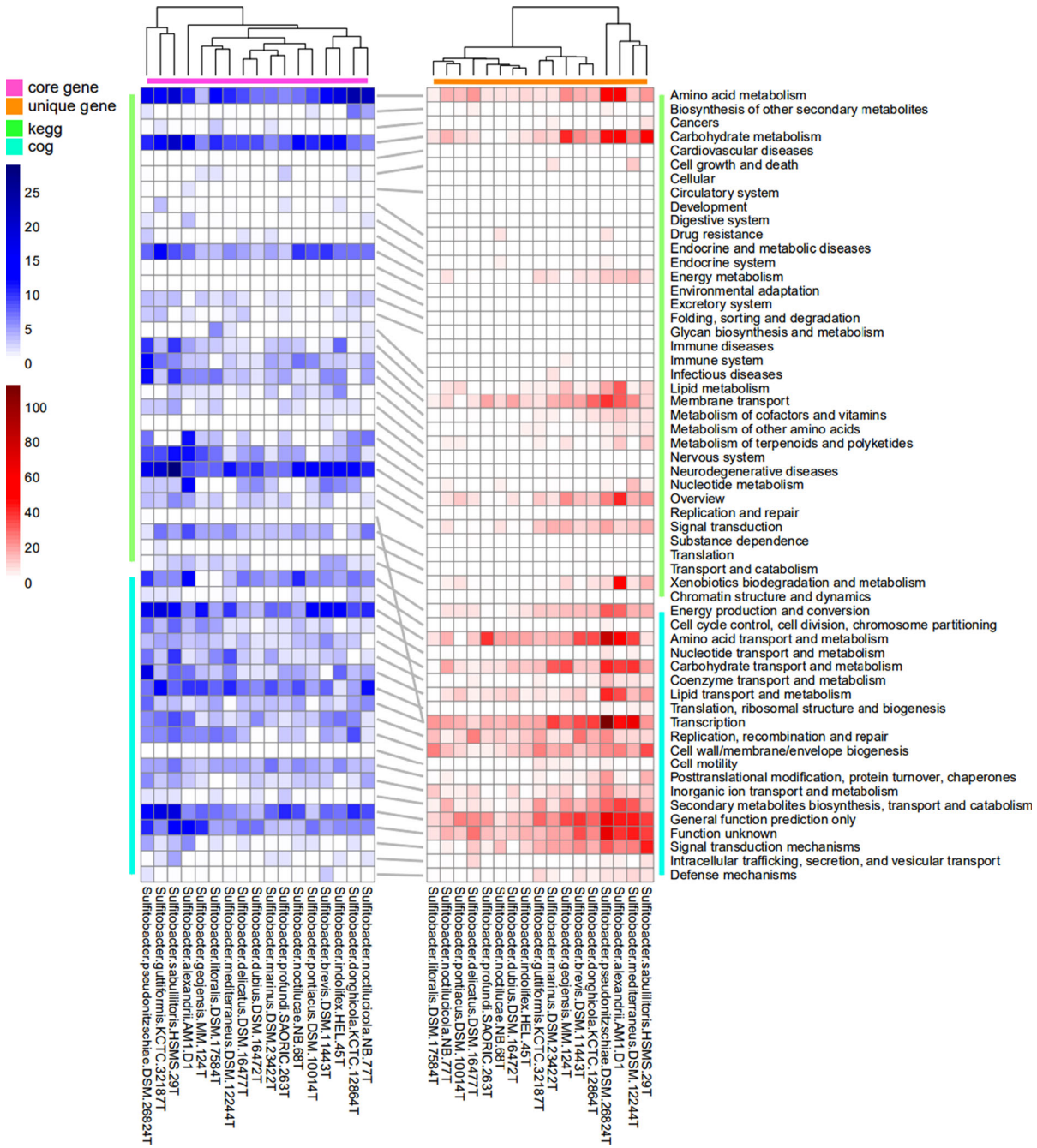

Fig. 6 Comparison of the core genes, unique genes, KEGG and COG characteristics of the genomes between strain AM1-D1^T and the other 16 type strains of *Sulfitobacter* with available genomes

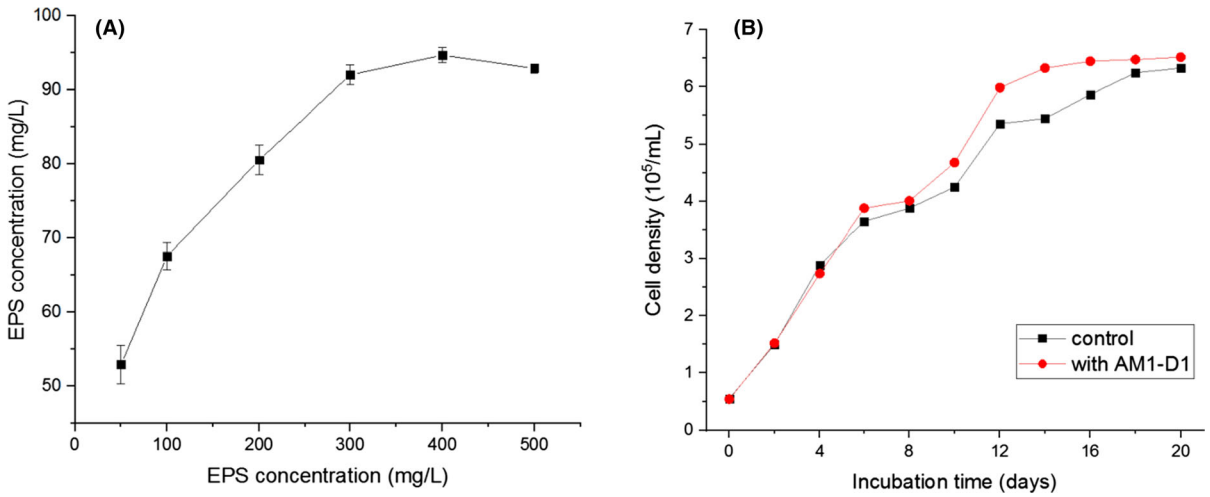

Fig. 7 The bioactivity evaluations by the bioflocculanting assay of bacterial exopolysaccharides (EPS) produced by strain AM1-D1^T (**a**), and microalgae growth-promoting potential assay (**b**)

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Author contributions QY and XLZ conceived the project and designed the experiments; QY and YMG performed the experiments; QY, NMI, XY and XLZ analyzed the data; QY and XZ drafted and revised the manuscript. All authors have read and approved the final version of the manuscript.

Availability of data and materials Strain AM1-D1^T has been deposited in two independent international culture centers (CCTCC in China, and KCTC in South Korea) with the deposition no. CCTCC AB 201696 and KCTC 52626, respectively.

Declarations

Conflict of interest All the authors have declared no conflict of interest.

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