Sulfuriroseicoccus Oceanibius Gen. Nov., Sp. Nov., A Representative of The Phylum *Verrucomicrobia* With A Special Cytoplasmic Membrane and A Proposal for *Sulfuriroseicoccaceae* Fam. Nov.

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Research Article

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Abstract

Here, we describe a novel bacterial strain from marine sediment, designated T37^T, which was isolated from the marine sediment of Xiaoshi Island, PR China. Growth of strain T37^T occurs at 15–40 ℃ (optimum, 37 ℃), pH 6.0–9.0 (optimum, 7.5), and in the presence of 0.5–5.5 % (w/v) NaCl (optimum, 1.5 %). Characteristic phenotypic traits of the novel strain include MK-9 as the major menaquinone. The major fatty acids identified were iso-$C_{14:0}$ and $C_{16:1}$ $\omega 9c$. Phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and phosphoglycolipids were the major cellular polar lipids. The G + C content of genomic DNA was 58.4 mol%. Unusual outer membrane features deduced from the analysis of cell morphology could have formed an enlarged periplasmic space putatively used for the digestion of macromolecules. Phylogenetic analyses based on 16S rRNA genes and the genome indicated that strain T37^T represents a novel species and genus affiliated with a distinct family level lineage of the verrucomicrobial subdivision 1. Our polyphasic taxonomy approach places the novel strain in a new family within the class *Verrucomicrobiae*, for which the name *Sulfuriroseicoccaceae* fam. nov. is proposed. Strain T37^T (= KCTC 72799^T = MCCC 1H00391^T) is the type strain, for which the name *Sulfuriroseicoccus oceanibius* gen. nov., sp. nov. is proposed.

Introduction

Verrucomicrobia play important roles in natural ecosystems, such as soil, marine, freshwater, and even in the human gut. The phylum *Verrucomicrobia* was originally defined in 1997 (Hedlund et al. 1997). However, it was recognised by the application of 16S rRNA gene sequencing in 1987 (Albrecht et al. 1987). *Verrucomicrobium spinosum* was the first to be sequenced, and a new bacterial division was suggested as the most suitable for its diverse phylogenetic position relative to other bacteria. Later, Hedlund established formal class-, order-, and family-level taxonomic categories. The phylum *Verrucomicrobia* has historically been divided into at least five subdivisions. Many members of *Verrucomicrobia* are distant and enough to be different phyla. One species in subdivision 5 has been proposed as a separate new bacterial phylum *Kiritimatiellaeota* (Spring et al. 2016), and *Victivallis vadensis*, originally viewed as a member of subdivision 7, is now known to be a member of the separate phylum *Lentisphaerae* (Cho et al. 2004).

Verrucomicrobia is highly diverse in cultivation-independent surveys based on the recovery of 16S rRNA gene sequences from many different habitats. They have evolutionary and cellular significance regarding their relationship with complex cell structures and an intriguing presence of close homologs to eukaryotic proteins, such as tubulins (Jenkins et al. 2003). *Verrucomicrobia* is also a medically important phylum, and includes species such as *Akkermansia muciniphila*, which may play key roles in the microbial communities in humans (Depommier et al. 2019). Additionally, they are of immense significance to the understanding of ecology and their function, such as members of the genus *Methyloacidiphilum*, which are important in the elucidation of bacterial methane oxidation (Hou et al. 2008). *Verrucomicrobia* is a culturable representative species of the rare but very valuable phylum. However, to date, few

representatives have been cultivated, and most have yet to be cultured and described. At the time of writing, only 60 species are (validly) described based on LPSN (LPSN, List of Prokaryotic Names with Standing in Nomenclature) (Parte et al. 2020). However, the doughnut-shaped pits in the outer membrane of the *Gemmataceae* and *Isosphaeraceae* of *Planctomycetes* are a unique hallmark trait. It has been reported that the periplasmic space of *Planctomycetes* can be greatly enlarged and convoluted for macromolecule uptake (Boedeker et al. 2017). Here, we present the description of *Sulfuriroseicoccus oceanibius* T37^T (= KCTC 72799^T = MCCC 1H00391^T), a novel genus, *Sulfuriroseicoccus*, and species in a novel family, *Sulfuriroseicoccaceae* fam. nov., is proposed to accommodate the genus, in which doughnut-shaped pits were found in the outer membrane as a unique hallmark trait of this strain.

Material And Methods

Isolation and cultivation

We considered that culturing highly diverse bacteria is indeed possible when their ecological niche is sufficiently well mimicked. We then designed the “medium S” for cultures. The basal marine medium was designed in this study to match the salinity of seawater, which contained 80 % natural seawater, 20 % artificial seawater (consisting of 3.5 % NaCl, 0.32 % $MgSO_4$, 0.22 % $MgCl_2$, 0.12 % $CaCl_2$, 0.07 % KCl, 0.02 % $NaHCO_3$, and 1.0 % $Na_2S_2O_3$), and 100 g l^{-1} fresh sediment. After filtering with gauze, filtrates were obtained. For carbon and nitrogen sources, the initial cultivation medium for marine bacterial strains was supplemented with 0.33 g L^{-1} sodium pyruvate, 0.5 g L^{-1} peptone (Hopebio), and 0.1 g L^{-1} yeast extract (Hopebio). The pH of the medium was adjusted to 7.5 with NaOH. All medium components were sterilised by autoclaving, except for $NaHCO_3$, which was filtered through a 0.22 μM pore polyethersulfone filter.

Sediment samples were collected from Xiaoshi Island (37°31' 36" N, 122°00' 58" E), Weihai, PR China, which is a national marine nature reserve. The sediment sample was collected from approximately 10 cm depth in the intertidal zone after high tide, in November 2018. The sample was serially diluted to 10^{-3} with sterilised seawater and 0.1 mL aliquots of each dilution were spread onto 1/10-strength marine medium using 2216 (MA; BD) plates as the basal medium. The plates were incubated in an aerobic environment for 30 d at 25 ℃. After incubation, a tiny pink colony was selected from the plate. After repeated streaking, the strain was routinely cultivated on MA at 30 ℃ for 10–15 d. The strain was stored in sterile 15 % (v/v) glycerol supplemented with 1 % (w/v) NaCl suspensions at -80 ℃. The novel bacterium, designated T37^T, was isolated and selected for detailed taxonomic analyses, and strain T37^T was cultivated on MA at 37 ℃ unless otherwise mentioned.

Phylogeny-based on 16S rRNA gene sequences

To classify this bacterium, the 16S rRNA gene was amplified using primers 27F and 1492R (Lane 1991). Purified PCR products were sequenced by BGI Co., Ltd. (Qingdao, China), and a mostly complete 16S rRNA gene sequence was obtained. The mostly complete 16S rRNA gene sequence of strain T37^T was preliminarily identified by searching for matches in the EzBioCloud (http://www.ezbiocloud.net) and

GenBank databases (https://www.ncbi.nlm.nih.gov) for further phylogenetic analysis. Comparative sequence analyses of the mostly complete 16S rRNA gene sequences were used to determine the phylogenetic position of strain T37^T to related strains.

To determine its exact taxonomic status, we performed further phylogenetic analyses. The 16S rRNA gene sequences of the novel strains, all the published verrucomicrobial strains and the uncultured strains, which the 16S rRNA gene sequence similarities were higher than the 86.5 % family threshold identity, were collected. Additionally, 16S rRNA gene sequences from genome sequences were extracted using RNAmmer version 1.2 (Karin et al. 2007) and compared with the 16S rRNA gene reference database from phylogenetic trees through the analysis of BLAST sequence alignment to ensure authenticity. Next, according to similarity and completeness, duplicate 16S rRNA genome sequences were discarded from the study. Finally, the 16S rRNA sequences of 125 strains were obtained. Using the MUSCLE program (Edgar 2004), trimAl (Capella-Gutierrez et al. 2009) was used to automatically remove heterotypic sequences and differential regions according to the length and number of ambiguous bases. A comprehensive sequence alignment was generated using sequences extracted from genomic sequences or 16S rRNA gene sequences previously obtained from type strains. Based on the 16S rRNA gene sequence, the phylogenetic tree analysis was reconstructed using the FastTree 2 program (Price et al. 2010) and RAxML version 8 (Alexandros 2014) and visualised using MEGA version 7.0 (Sudhir et al. 2016). Bootstrap analysis was performed with 1,000 replications to provide confidence estimates for tree topologies (Felsenstein and Joseph 1985).

Genome sequencing, annotation, and analysis

The biomass of strain T37^T was collected, and genomic DNA was extracted according to the instructions of the genome extraction kit (TAKARA). The extracted DNA was detected by agarose gel electrophoresis for degradation and contamination, and a microspectrophotometer (Nanodrop, Thermo Fisher Science and Technology) was used to determine the extracted genomic DNA content, to ensure the content of at least 12 μg. Using the extracted genomic DNA as a template for the PCR, the 16S rRNA gene sequence was amplified and sequenced, and the sequencing results were verified by BLAST comparison. After the bacterial strain information was confirmed, the extracted genomic DNA was sent to Tianjin Novozhiyuan Bio-Information Technology Co., Ltd. for genome sequencing. The PacBio platform and Illumina platform were used for library construction and inspection. After qualified library inspection, PacBio Sequel and Illumina Novaseq PE150 were used for sequencing and genome assembly was completed using SMRT Link Version5.0.1 software (Simon et al. 2018). Protein-encoding regions were identified using the Cluster of Orthologous Group of Proteins (COG) (Tatusov et al. 2003). Gene content was annotated using the NCBI Prokaryotic Genome Annotation Pipeline, and the genes involved in metabolic pathways were analysed in detail using information from the KEGG database (Minoru et al. 2016). The secondary metabolic gene clusters were predicted by antiSMASH (Medema et al. 2011), active carbohydrate enzymes were predicted by Cazy (Carbohydrate-Active enZYmes Database) (Cantarel et al. 100AD), resistance genes were predicted by Comprehensive Antibiotic Research Database (CARD) (Alcock et al.

2019), and membrane transporters were predicted by Transporter Classification Database (TCDB) (Saier et al. 2006).

From the EzBioCloud (https://www.ezbiocloud.net) and NCBI databases (https://www.ncbi.nlm.nih.gov) all the genome of published verrucomicrobial strains were downloaded. Then, the bacterial core gene set coverage was used to examine genome integrity, determine whether the genome is polluted compared to the ContEst16S (Lee et al. 2017), and retain genomes that have fewer than 500 contigs, completeness of 95.0 % or greater, and no contamination. For the genomic phylogenetic tree, UBCG (Na et al. 2018) was used to analyse the phylogenetic relationships. Based on 49 genomes, FastTree 2 (Price et al. 2010) using GTR + CAT parameters and IQtree (Jana et al. 2016) using the GTR + F + I + G4 model was used to reconstruct the phylogenetic tree, and 1000 bootstrap replicates were used for analysis. Phylogenetic analysis was performed using MEGA version 7.0 (Sudhir et al. 2016).

The genome sequences of the strains of the order *Verrucomicrobiales* were obtained from the NCBI database. Calculations of genomic G + C content values and genomic size were also consistent with the taxonomic mill (García-López et al. 2019). The genomic sequences of 22 strains were analysed for their related species, including the average amino acid identity (AAI) (Rodriguez and Konstantinidis 2014), percentage of conserved proteins (POCP) (Qin et al. 2014), average nucleotide identity (ANI) (http://jspecies.ribohost.com/jspeciesws/) (Yoon et al. 2017), and the digital DNA-DNA hybridisation (dDDH) (http://ggdc.dsmz.de/distcalc2.php) (Goris et al. 2007) were determined. However, we focused on the genetic relationships among different genera; therefore, we focused on analysing the AAI values between the genomes. The AAI calculator estimates, which were calculated using Enve-omics (http://enve-omics.ce.gatech.edu/aai/index), using both best hits (one-way AAI) and reciprocal best hits (two-way AAI) between two genomic datasets of proteins. The AAI values were set at a threshold of 60.0 % as the boundary of the genus (Rodriguez and Konstantinidis 2014).

Cell morphological analysis

The determination of the cell characteristics of strain T37^T was performed using cells grown on MB for 3 d at 37 ℃. Cell division, morphological analysis, and size were examined by light microscopy (Carl Zeiss Axioscope A1), scanning electron microscopy (model Nova NanoSEM450; FEI), and transmission electron microscopy (JEM1200, Japan).

The cells were collected and diluted 1000 times (OD_{600} = 1.0), washed twice with 0.1 mol/L phosphoric acid buffer (PBS) at pH 7.0, and resuspended in 200 μL staining solution FM4-64/water = 1:19; the final concentration of FM4-64 was 3 μg/mL, and the mixture was incubated at room temperature for 10 min. The cells were resuspended in 200 μL staining solution DAPI:water (1:25), the final concentration of DAPI was 1 μg/mL, and the mixture was incubated at room temperature for 10 min. The cells were washed twice with PBS and centrifuged at 2,500 × *g* for 1.5 min. Finally, the cells were resuspended in 200 μL PBS. During the entire process, the cells were observed and photographed using a fluorescence microscope. The FM4-64 dye stains cell membrane lipids and emits red fluorescence (maximum

excitation/emission wavelength is approximately 515/640 nm) (Amerik and Hochstrasser 2006), and DAPI is a fluorescent dye that strongly binds to DNA (Piotr et al. 2001). According to the intensity of fluorescence, the amount of DNA can be determined by the amount of blue light emitted (maximum excitation/emission wavelength is approximately 340 nm/488 nm).

Transmission electron microscopy of ultrathin sections of strain $T37^T$ was performed as described by Pimentel-Elardo et al. (Pimentel-Elardo et al. 2003). For transmission electron microscopy, cells were grown in liquid culture and fixed with 2.5 % glutaraldehyde for 24 h at 4 ℃, washed three times with PBS (0.1 M), pH 7.0. In the fume hood, the sample was fixed with 1 % osmium acid solution for 1–2 h, the osmium acid waste solution was carefully removed, and the sample was washed three times with 0.1 mol/L PBS buffer at pH 7.0, 15 min each time. The samples were dehydrated with increasing concentrations of ethanol (from 30 to 100 %) for 15 min at each concentration and then treated with 100 % ethanol for 20 min. A drop of culture was incubated on a copper grid, the liquid was removed, and the cells were stained with a drop of 0.5 % uranyl acetate for 5 min. The cells were examined using transmission electron microscopy (JEM1200, Japan).

For scanning electron microscopy, colonies with surrounding material were fixed in 2.5 % glutaraldehyde for 4 h at 4 ℃, washed three times with 0.1 M PBS, pH 7.0, and dehydrated with increasing concentrations of ethanol (from 30 to 100 %) for 10 min. After critical-point drying and platinum coating of the dried material, the colonies were examined using scanning electron microscopy (model Nova NanoSEM450; FEI). Before photographing, it was important to select the field of view where the cells did not overlap and block each other. Additionally, it was necessary to select a relatively high magnification to view single cells.

Physiological tests

The temperature range for growth was determined on MA at 4–45 ℃. The NaCl concentrations for growth were determined by incubating the bacteria in modified marine broth 2216 made with 0.5 % peptone, 0.1 % yeast extract, and artificial seawater (0.32 % $MgSO_4$, 0.22 % $MgCl_2$, 0.12 % $CaCl_2$, 0.07 % KCl, 0.02 % $NaHCO_3$, w/v) in the presence of 0.0–10.0 % (w/v) NaCl at intervals of 0.5 %. The effects of pH were determined by adding the appropriate buffers (Sangon), including MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5), and CAPSO (pH 9.0–9.5) to MB at a concentration of 20 mM, and the pH of the control groups was checked after autoclaving. The OD_{600} values of the cultures were measured after incubation for 3 d at 37 ℃. Cells of strain $T37^T$ were obtained from cultures grown on MA at 37 ℃ for 3 d, and the following phenotypic tests were performed as described in previous studies (Feng et al. 2020). Motility was determined by the hanging-drop method, and gliding motility was tested by inoculating the bacteria on 0.5 % agar. The Gram reaction was determined using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Growth under anaerobic conditions (10 % H_2, 5 % CO_2, and 85 % N_2) was determined after incubation for 14 d in an anaerobic chamber with or without 0.1 % (w/v) KNO_3. Susceptibility to antibiotics was investigated on MA using the disc diffusion method, and according to procedures outlined by the Clinical and Laboratory

Standards Institute (Cockerill et al. 2011). Catalase activity was determined by observing bubble formation in a 3 % H_2O_2 solution. Oxidase activity was examined using an oxidase reagent kit (bioMérieux) according to the manufacturer's instructions. Tests were performed for the hydrolysis of starch, casein, alginate, carboxymethylcellulose, and Tweens 20, 40, 60, and 80. All experiments were performed in triplicates. Biochemical tests were performed using API 50CHB (bioMérieux) kits (http://www.biomerieux-diagnostics.com/apir-id-strip-range) and API 20E (bioMérieux) kits were performed according to the manufacturer's instructions, using the biomass of strain T37^T grown on MA at 37 °C for 3 d. Production of other enzymes was assessed using API ZYM kits (bioMérieux). Carbon source oxidation was checked using BIOLOG GEN III microplates (http://www.biolog.com/). All API tests were performed according to the manufacturer's instructions, except that the salinity was adjusted to 1.5 %. All API and BIOLOG tests were performed with two replicates.

Chemotaxonomic characterizations analysis

Biomass for the study of chemotaxonomic features of strain T37^T was obtained from cultures grown in MB for 3 d (late logarithmic phase). Polar lipids were determined using 2D thin-layer chromatography (TLC) (Minnikin et al. 1982). Four separate TLC plates (EMD Millipore, 1.16487.0001) were prepared for each sample and individually stained using phosphomolybdic acid solution (total lipids), molybdenum blue solution (phosphates), α-naphthol sulfuric solution (carbohydrates), and ninhydrin (amines); all reagents were from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Isoprenoid quinones of strain T37^T were analysed using reverse-phase HPLC (Kroppenstedt and Reiner 1982). The preparation and extraction of fatty acid methyl esters from biomass and their subsequent separation and identification by gas chromatography were performed as previously described (Athalye et al. 2010). The fatty acids were extracted according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.1), methylated, and analysed using an Agilent 6890N gas chromatograph. Cellular fatty acids were identified using the TSBA40 database of the microbial identification system.

Results

Isolation and identification of strain

To isolate novel strains of *Verrucomicrobia*, sediment samples were collected from Xiaoshi Island (37°31' 36" N, 122°00' 58" E), Weihai, PR China, which is a national marine nature reserve. The "medium S" was designed for cultures and the obtained colonies were screened by 16S rRNA gene sequencing analysis. A novel strain was identified as a member of *Verrucomicrobia* based on pairwise comparison of 16S rRNA gene sequences of species in the EzBioCloud and NCBI databases.

Phylogenetic placement of strain T37^T

The 16S rRNA gene sequence of strain T37^T was previously determined and deposited in the GenBank/EMBL/DDBJ databases under the accession number MN412654. Based on the pairwise

comparison of 16S rRNA gene sequences of species in the EzBioCloud server (http://ezbiocloud.net/eztaxon), strain T37^T showed low levels of 16S rRNA gene sequence similarities to known members of the order *Verrucomicrobiales* and exhibited the highest sequence similarity with the uncultured strain SBYB-1201 (89.5 %), followed by uncultured strain sediment_deep44 (89.2 %), uncultured strain RESET_18A06 (88.9 %), *Roseibacillus persicicus*YM26-010^T (88.8 %), and *R. ishigakijimensis* MN1-741^T (88.8 %). The sequence of strain T37^T showed a maximum identity of 88.8 % within the cultured type strains of the *Verrucomicrobiaceae* family. The identity was higher than the 86.5 % family threshold identity (Yarza et al. 2014) but was below the 92.3 % family median identity. Following 16S rRNA-based taxonomic identity, the family rank seems appropriate for this strain. To determine its exact taxonomic status, we performed further phylogenetic and taxonomic investigation. Phylogenetic analysis of the 16S rRNA gene sequences performed using MEGA version 7.0 showed that strain T37^T formed a distinct lineage with the uncultured family GU230460 within the order *Verrucomicrobiales* (Fig. 2).

Genome characteristics

The genome was 3,496,488 bp long with 58.4 mol% GC content (Table 1). It was composed of one contig. Of the 2,855 predicted genes, 2,772 were protein-coding genes, and 71 were RNAs (including 15 rRNA, 52 tRNA, and four other RNA genes). A total of 1,917 genes (69.2 %) were assigned a putative function using NR BLAST. The remaining genes (n = 749) were annotated as hypothetical proteins (27.0 %). The properties and statistical information of the genome are presented in Table 1.

Phylogenetic analysis of the genomic sequences performed with the IQtree showed that strain T37^T formed a distinct lineage within the order *Verrucomicrobiales* (Fig. 3), which supported that strain T37^T could be assigned to a novel species of a novel family. The degree of genomic similarity of strain T37^T closely related species was estimated using average amino acid identity (AAI) analysis. Values among closely related taxa ranged from 55.9 % between strain T37^T and the order *Verrucomicrobiales* to 58.3 % (Fig. 4). When the isolate was compared to these close species, the highest value was 58.3 % with *R. squalenifaciens* DSM 18772^T, which was much lower than the boundary for the genus (60.0 %). Additionally, the POCP, ANI, and dDDH were determined, and the values were lower than the threshold (POCP<50.0 %, ANI<95.0 %, dDDH<70.0 %) (Table S1). Based on these results, the strain should be classified as a new family member.

The distribution of genes into COG functional categories is shown in Fig. S3. Notably, the highest proportion of genes was assigned to COG categories, including amino acid transport and metabolism [E], carbohydrate transport and metabolism [G], cell wall/membrane/envelope biogenesis [M], and translation, ribosomal structure, and biogenesis [J]. The proportion of genes in the mobilome: prophages and transposons [X] of strain T37^T was higher than that of other strains (Fig. 5). Moreover, it is a typical characteristic of free-living and chemoheterotrophic members of the PVC superphylum, which are often specialised in the degradation of recalcitrant polysaccharides (Manuel et al. 2012). Carbohydrate-active

enzymes were predicted using the CAZy database, and high proportions of genes assigned to CAZy (Fig. S4) were involved in glycoside hydrolases. Prediction of secondary metabolic gene clusters was performed using antiSMASH, and it was found that three gene clusters in strain T37^T were responsible for the biosynthesis of secondary metabolites: two gene clusters belonged to terpene and one gene cluster belonged to T3PKS. However, the structure of the secondary metabolites encoded by these three gene clusters could not be inferred. The strain T37^T also harbours a type 2 secretion system (T2SS), two type 3 secretion system (T3SS), and several putative prophages. The strain T37^T chromosome harbours genes associated with known drug resistance to fluoroquinolone (*mfd*, *gyrA*), rifampicin (*rpoB*), isoniazid (*katG*), and efflux pumps conferring antibiotic resistance (*abcA*, *adeCG*, *TaeA*). The genomes of strain T37^T contained approximately 125 membrane transport protein-related genes (Fig. S5).

Based on the annotated genome sequence, genes encoding proteins involved in the oxidative response (*batA*) and a major virulence factor (coproheme decarboxylase-HemQ) (Meuric et al. 2010) were found in the genome of strain T37^T. It may be an important trait for cells of strain T37^T to cope with toxic oxygen. FtsZ is an almost universal tubulin homologue in bacteria (Addinall and Holland 2002; Vaughan et al. 2004). Gene analysis of strain T37^T and related strains using the NCBI Prokaryotic Genome Annotation Pipeline, *Brevifollis gellanilyticus* NBRC 108608^T, *Prothecobacter fusiformis* ATCC 25309^T, *P. dejongeii* FC1^T, *P. debontii* FC3^T, and *P. vanneervenii* FC2^T differ from other bacteria in that they do not have the cell division protein of FtsZ but rather have tubulins. Neither tubulin nor the FtsZ homolog was found in the genomes of strain T37^T. It was speculated that the tubulin or FtsZ homologue sequence in the genome of the strain was not annotated because of the low similarity between the tubulin or FtsZ homologue sequence. Sulphite reductase (NADPH) was found, and it was suggested that the addition of $Na_2S_2O_3$ to the isolating medium might be helpful for the isolation of strain T37^T. PVC bacteria are generally believed to be susceptible to antibiotics that target protein syntheses, such as chloramphenicol, tetracycline, and erythromycin, or those that target DNA replication, such as fluoroquinolones (C. et al. 2010; Godinho et al. 2019). However, the results are different from those previously reported, and strain T37^T was resistant to ofloxacin and norfloxacin, and the resistance genes (*mfd* and *gyrA*) to fluoroquinolone were detected.

Cell morphology and physiology

After 3 d of incubation at 37 °C on MA, the cells of strain T37^T grew in various forms, including almost coccoid cells ranging from 0.5 to 1.0 μm in diameter and rod-shaped cells ranging in width from 0.4 to 0.9 μm and in length from 0.5 to 1.5 μm (Fig 1a). We speculated that the short rod-shaped cells could be divided. To verify this hypothesis, the growth curve was determined, and the cells of strain T37^T at different growth periods were observed. We found that in each period, coccoid and rod cells could be observed simultaneously (as shown in Fig. S1). Single cells and cell clusters were also identified. Motile cells, flagella, and prostheses were not observed. Cytoplasmic invagination was observed in the cells of strain T37^T (Fig. 1b-c, h). Additionally, differentiation within cells was observed in the transmission thin section cells, and the typical features of cells with typical bacteria were visible, such as the plasma membrane (CM) and cell wall of nucleoid cells (Fig. 1g). The strain cells had a major cytoplasmic intima

(ICM) along their surface next to the cytoplasmic membrane (Fig. 1g). The cells had two major compartments. A larger compartment, between which the membrane was similar to the characteristics of the floating bacterium cell program, and a smaller endoplasmic compartment, with the cytoplasm located between the ICM and CM.

Based on a literature research, cells of some type strains of *Verrucomicrobia* also were coccoid or rod-shaped, such as *L. cuticulihirudinis* E100^T (Glaeser et al. 2012), *Rubritalea spongiae* YM21-132^T, *R. tangerina* YM27-005^T [52], *R. marina* DSM 17716^T (Scheuermayer et al. 2006), and *R. sabuli* YM29-052^T [53]. Generally, there are two main reasons for the change in bacterial cell shape from rod-shaped to coccoid. Cells would change from rod-shaped to coccoid because they are missing the mutation of MreB protein, such as the cells of *Bacillus subtilis* changed from rod-shaped to coccoid. It has been shown that the structural changes of penicillin-binding proteins (PBPs) caused by the functional defects of PBPs, can cause changes in cell morphology, especially the key control protein penicillin-binding protein 2 (penicillin-binding protein 2). Only the genomic information of strain *R. marina* DSM 17716^T was obtained, and the genome of strain *R. marina* DSM 17716^T was analysed using the genome of strain T37^T. Genome analysis revealed that strain *R. marina* DSM 17716^T and strain T37^T only had the MreC protein and lacked the MreB protein. It was also possible that some cells of strain *R. marina* DSM 17716^T and strain T37^T were coccoid from short rod-shaped because of the deletion mutation of the MreB protein.

Doughnut-shaped pits were found in the outer membrane of *Gemmata obscuriglobus* of *Planctomycetes*, and there is a receptor-mediated protein uptake mechanism (Lonhienne et al. 2010), suggesting that these bacteria might possess a simple form of endocytosis. However, the hypothesis of endocytosis is clearly refuted in a study by Boedeker and the cytoplasmic membrane can be formed by an enlarged periplasmic space putatively used for the digestion of macromolecules (Boedeker et al. 2017). From the similarities between *Planctomycetes* and *Verrucomicrobia*, which belong to the PVC superphylum (Wagner and Horn 2006), it is speculated that the cytoplasmic membrane of strain T37^T may be used for the digestion of macromolecules. The most representative electron micrographs were selected from at least two independent experiments and more than 50 cells were analysed. The doughnut-shaped pits were found in the outer membrane of the cells of strain T37^T (Fig. 1b-c, h). A series of pre-treatments were conducted before the bacterial cells were observed by electron microscopy, which might affect cell morphology. Therefore, the same pre-treatments were applied to strain T37^T and *Escherichia coli* DH5α for scanning electron microscopy and transmission electron microscopy. FM4-64 and DAPI were used to stain the cells of *E. coli* DH5α and strain T37^T, and fluorescence microscopy was used to observe the cells of *E. coli* DH5α and strain T37^T after the same staining treatment. As a control, no pits appeared on the outer membrane of *E. coli* DH5α cells, and the DNA (shown in blue) of *E. coli* DH5α cells (Fig. 1f) was distributed evenly throughout the cells. From the fluorescence microscope image of cells of strain T37^T (Fig. 1e), the DNA of strain T37^T was clustered on one side of the cell, and the cell membrane of cells of strain T37^T (shown in red) was not uniformly distributed. Based on the above observation results, it can be confirmed that the surface of strain T37^T probably has pits.

Physiology

Strain T37^T was Gram-stain-negative, facultatively anaerobic, non-motile, and coccoid. Colonies on MA were round, light pink, and glossy with entire margins. Growth occurred in 0.5–5.5 % (w/v) NaCl (optimum, 1.5 %), 15–40 ℃ (optimum, 37 ℃), and at pH 6.0–9.0 (optimum, pH 7.5). Strain T37^T reduced nitrate to nitrite. Cells of strain T37^T were catalase-negative and oxidase-positive (weakly). The strain was positive for starch hydrolysis, but negative for casein, Tweens 20, 40, 60 and 80, alginate, cellulose, and DNase. Strain T37^T was susceptible to lincomycin (2 μg), chloramphenicol (30 μg), clarithromycin (15 μg), and erythromycin (15 μg), but resistant to carbenicillin (100 μg), penicillin (30 μg), ceftriaxone (30 μg), ampicillin (10 μg), rifampin (5 μg), cefotaxime sodium (30 μg), ofloxacin (5 μg), gentamycin (10 μg), tetracycline (30 μg), kanamycin (30 μg), tobramycin (10 μg), vancomycin (30 μg), neomycin (30 μg), norfloxacin (10 μg), and streptomycin (10 μg). The phenotypic characteristics determined by the API and BIOLOG tests are provided in the species description. Other phenotypic characteristics from classical experiments are listed in the species description, whereas the comparative analyses of phylogenetically related taxa are given in Table 2.

Chemotaxonomic characterizations

The menaquinone detected in strain T37^T was MK-9. A list of the fatty acid compositions of strain T37^T was presented in Table 3. The major cellular fatty acids (>10.0 %) in strain T37^T were iso-$C_{14:0}$ (47.5 %) and $C_{16:1}$ $\omega 9c$ (13.6 %), $C_{16:0}$ was not the main component that could be used to distinguish strain T37^T from the closely related strains based on the type and proportion. The polar lipids consisted of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphoglycolipids, phosphatidylcholine, phosphatidylinositol, one unidentified aminolipid, an unidentified phospholipid, and four unidentified lipids (Fig. S2). Strain T37^T contained unsaturated fatty acids and branched-chain fatty acids as the major cellular lipids, and many kinds of phospholipids were the major polar lipids.

Discussion And Conclusion

The novel strain T37^T was isolated from sediment and represented a novel family within the order *Verrucomicrobiales*. Characterisation of the first cultured representative of the *Verrucomicrobia* subdivision 1 revealed the lifestyle of a facultatively anaerobic bacterium with cytoplasmic invaginations of the internal membrane. We speculate that the cytoplasmic membrane can form an enlarged periplasmic space putatively used for the digestion of macromolecules. It may constitute useful models for future experimental tests at the molecular level for how endogenous membrane invagination could give rise to endomembrane systems and the nucleus.

Furthermore, the genus *Sulfuriroseicoccus* has not been provided with any definitive family within the order *Verrucomicrobiales*. The genus could be distinguished from the related families in the order *Verrucomicrobiale*s by several features (Table 2). Unlike members of the family *Verrucomicrobiaceae*, strain T37^T is not rod-shaped and is catalase-negative and oxidase-positive (weakly). In contrast to

members of the family *Rubritaleaceae*, strain T37^T could grow at 40°C. Members of the family *Akkermansiaceae* are strictly anaerobic, but strain T37^T is facultatively anaerobic. The four families could also be differentiated based on their chemotaxonomic features. Members of other families of the order *Verrucomicrobiales* have straight-chain fatty acids, unsaturated fatty acids, and branched-chain fatty acids as the major cellular lipids, whereas strain T37^T does not have straight-chain fatty acids as the major cellular fatty acids. Additionally, strain T37^T has phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and phosphoglycolipids as the major polar lipids.

Description of Sulfuriroseicoccus **gen. nov.**

Sulfuriroseicoccus [Sul.fu.ri.ro.se.i.coc'cus. L. neut. n. *sulfur* sulfur; L. masc. adj. *roseus* rosy, N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos* a berry) a coccus; N.L. masc. n. *Sulfurisoreicoccus* rosy sulfur-reducing coccus]

Cells are Gram-stain-negative, facultatively anaerobic, cocci-shaped. Catalase-negative and oxidase-positive (weakly). The predominant menaquinone is MK-9 and the major cellular polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphoglycolipidss. The main cellular fatty acids are iso-$C_{14:0}$ and $C_{16:1}$ $\omega 9c$. The genus belongs to the family *Sulfuriroseicoccaceae* of the order *Verrucomicrobiales*.

Description of Sulfuriroseicoccus oceanibius **sp. nov.**

Sulfuriroseicoccus oceanibius (O.ce.a.ni'bi.us. L. masc. n. *oceanus*, the ocean; Gr. masc. n. bios, life; N.L. masc. n. Oceanibius an ocean life)

Exhibits the following properties in addition to those given in the genus description. Cells are non-motile, non-flagellated, approximately 0.5-1.0 μm in diameter. Colonies on MA are round, light-pink and glossy with entire margins, about 1.0 mm in diameter after incubation for 3 days at 37 ℃. Growth occurs at 15–40 ℃ (optimum, 37 ℃), at pH 6.0–9.0 (optimum, 7.5) and in the presence of 0.5–5.5 % (w/v) NaCl (optimum, 1.5 %). Growth is not observed under anaerobic conditions on MA with or without 0.1 % (w/v) KNO_3. Nitrate is reduced to nitrite. Starch is hydrolyzed, but casein, Tweens 20, 40, 60, 80, alginate, cellulose and DNase are not. Positive for voges–proskauer reaction. The following substrates were oxidized: dextrin, d-trehalose, d-cellobiose, d-maltose, gentiobiose, α-d-lactose, sucrose, d-turanose, *N*-acetyl-*β*-d-Mannosamine, *N*-acetyl-d-glucosaminidase, *N*-acetyl-d-galactosamine, d-mannose, d-fructose, l-fucose, l-rhamnose, d-galacturonic acid, l-galactonic acid lactone, glucuronamide, methyl pyruvate, d-lactic acid methyl ester, d-malic acid, l-malic acid and α-keto-butyric acid. Positive for tryptophan deaminase, gelatinase, alkaline phosphatase, esterase (C4), leucine arylamidase, *N*-acetyl-*β*-glucosaminidase, acid phosphatase, naphthol-AS-BI-phosphphydrolase, but negative for esterase lipase (C8), lipase(C14), cystine arylamidase, valine arylamidase, trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *α*-mannosidase and *β*-fucosidase. Sensitive to lincomycin, chloramphenicol, clarithromycin and erythromycin, but resistant to carbenicillin,

penicillin, ceftriaxone, ampicillin, rifampin, cefotaxime sodium, ofloxacin, gentamycin, tetracycline, kanamycin, tobramycin, vancomycin, neomycin, norfloxacin and streptomycin.

The type strain is T37^T (= KCTC 72799^T = MCCC 1H00391^T), isolated from coastal seawater of Xiaoshi Island, Weihai, PR China. The complete genome size of strain T37^T was determined to be 3.5 Mbp and the DNA G + C content of the type strain is 58.4 mol%. The GenBank accession number for the 16S rRNA gene sequence of strain T37^T is MN412654 and for the draft genome sequence is CP066776.1.

Description of Sulfuriroseicoccaceae **fam. nov.**

Sulfuriroseicoccaceae (Sulfo.globus a.ce'ae. - N.L. masc. n. *Sulfuriroseicoccus* type genus of the family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Sulfuriroseicoccaceae*, the *Sulfuriroseicoccus* family).

The description of this family is the same as that given for the genus *Sulfuriroseicoccus*. The type genus of the family is *Sulfuriroseicoccus*. The family belongs to the order *Verrucomicrobiales* of the class *Verrucomicrobiae*.

Abbreviations

KCTC, Korean Collection for Type Cultures; DSMZ, Leibniz Institute German Collection of Microorganisms and Cell Cultures; MCCC, Marine Culture Collection of China; NBRC, NITE Biological Resource Center; BD, Becton Dickinson; MEGA, Molecular Evolutionary Genetics Analysis; MA, marine agar 2216; MB, marine broth 2216; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

Declarations

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Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships.

Author Contributions XF performed most of the experiments, wrote the main part of the manuscript, and functions as the first author. QHZ helped with the isolation of the novel strains and with cultivation measurements. MQY contributed to the literature research and analyzed the sequencing data. XF and ZJD designed the study and helped with experimental setups and design.

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Availability of data and materials The 16S rRNA gene sequence and the complete genome sequence of strain T37^T, is deposited in GenBank under accession number MN412654 and CP066776.1. The type strain T37^T can be obtained from the Korean Collection for Type Cultures (KCTC 72799^T) and the Marine Culture Collection of China (MCCC 1H00391^T).

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Tables

Table 1. Nucleotide content and gene count levels of genome

Attribute	Genome
GenBank ID	CP066776.1
Genome size (bp)	3496488
G+C content (%)	58.42
Polished contigs	1
N50 length(bp)	3513074
Total genes	2855
RNA genes	71
Protein-coding genes	2772
Genes with function prediction	2023
Genes assigned to COGs	1609
Genes assigned to NR BLAST	1917

COGs, Clusters of Orthologous Groups database.

Table 2. Characteristics that distinguish strain T37^T phylogenetically related taxa.

Taxa: 1, Strain T37^T; 2, *Roseibacillus ishigakijimensis* KCTC 12986^T [51]; 3, *Haloferula rosea* KCTC 22201^T [54]; 4, *Akkermansia muciniphila* ATCC BAA-835^T [13]; 5, *Rubritalea marina* DSM 17716^T [43]. +, Positive; w, weakly positive; –, negative, nd, not detected

Table 3. Fatty acid compositions of strain T37^T. Only fatty acids comprising ≥1.0% of the total are shown. Tr, fatty acids amounting (<1.0 %) are not shown. Major components (≥10.0 %) are given in bold

Characteristic	1	2	3	4	5
Cell shape	Coccoid or rod	Rod	Rod	Oval	Coccoid or rod
Colony colour	Pink	Pink	Pale-pink	White	Red
Tolerance to oxygen	+	+	+	-	+
Oxidase	w	+	+	nd	+
Catalase	-	+	-	nd	-
Temperature range (°C)	15-40	20-37	20-37	20-40	8-30
Growth pH	6.0-9.0	6.5-9.0	6.5-9.0	5.5-8.0	6.7-8.2
Menaquinones	MK-9	MK-9	MK-9	nd	MK-8, MK-9
Major fatty acids (>10 %)	iso-$C_{14:0}$, $C_{16:1}$ *ω9c*	iso-$C_{14:0}$, $C_{16:1}$ *ω9c*, $C_{16:0}$	iso-$C_{14:0}$, iso-$C_{16:0}$, anteiso-$C_{15:0}$	nd	iso-$C_{14:0}$, $C_{16:0}$, $C_{16:1}$ *ω7c* and/or 2-OH iso-$C_{15:0}$
DNA G+C content (mol%)	58.4	59.3	60.9	55.8	51.6

typeface.

Lipid type	Abundance (%)
Branched fatty acids	
iso-$C_{14:0}$	**47.5**
anteiso-$C_{15:0}$	4.2
iso-$C_{16:0}$	9.4
Unsaturated fatty acids	
$C_{15:1}\omega 8c$	6.8
$C_{16:1}\omega 9c$	**13.6**
$C_{17:1}\omega 8c$	1.5
$C_{18:1}\omega 9c$	tr
Straight-chain fatty acids	
$C_{14:0}$	3.3
$C_{16:0}$	5.4
$C_{17:0}$	1.7
$C_{18:0}$	tr

Figures

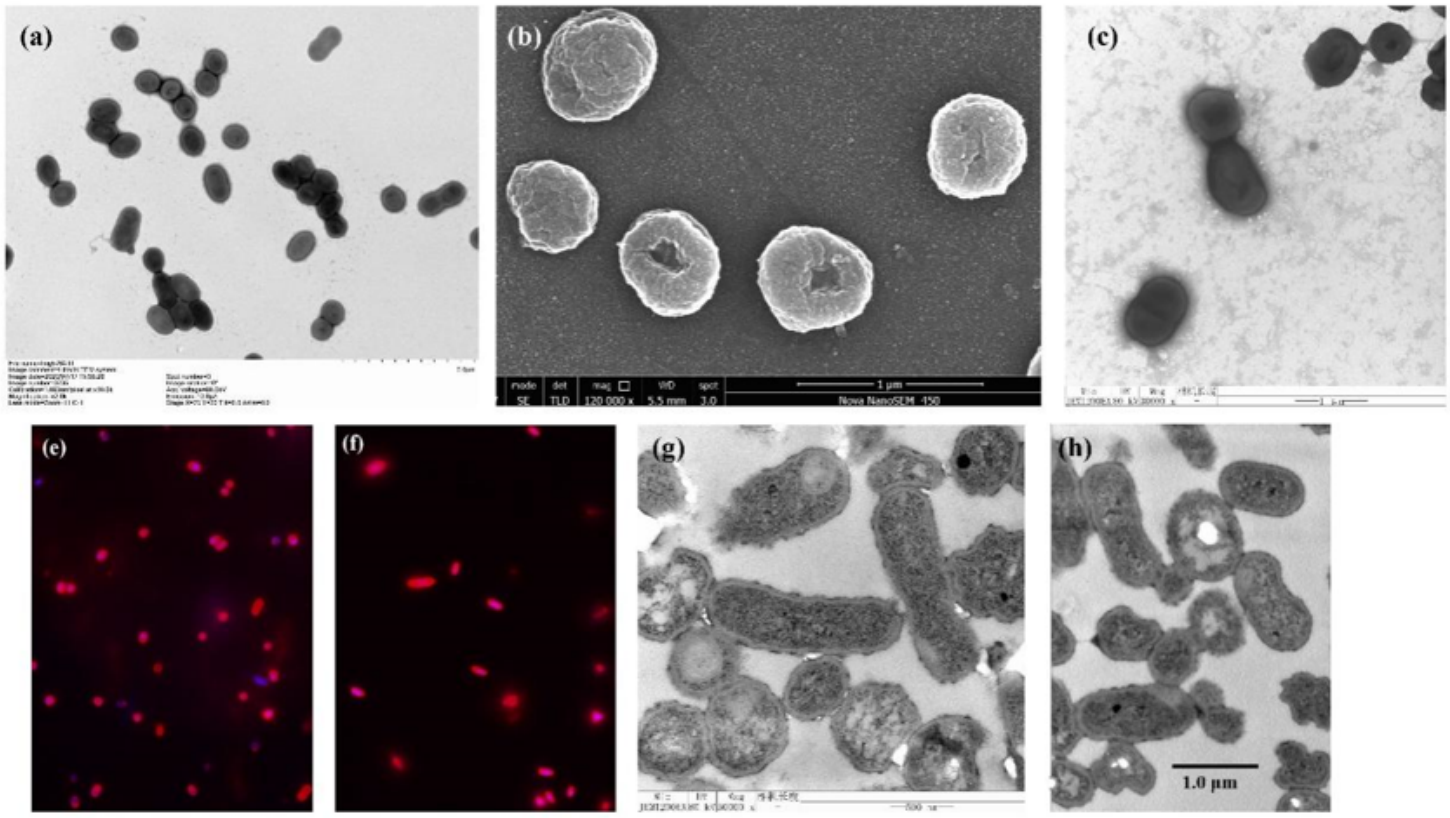

Figure 1

Shape and ultrastructure of cells of strain T37T. Cells were grown on MA at 37°C for 3 days. Scanning electron microscopy, transmission electron microscopy and transmission electron micrograph of a negatively stained thin section of cells of strain T37T. The selected micrographs originate from at least two independent experiments; more than 50 cells were analyzed and the best representative image was chosen.

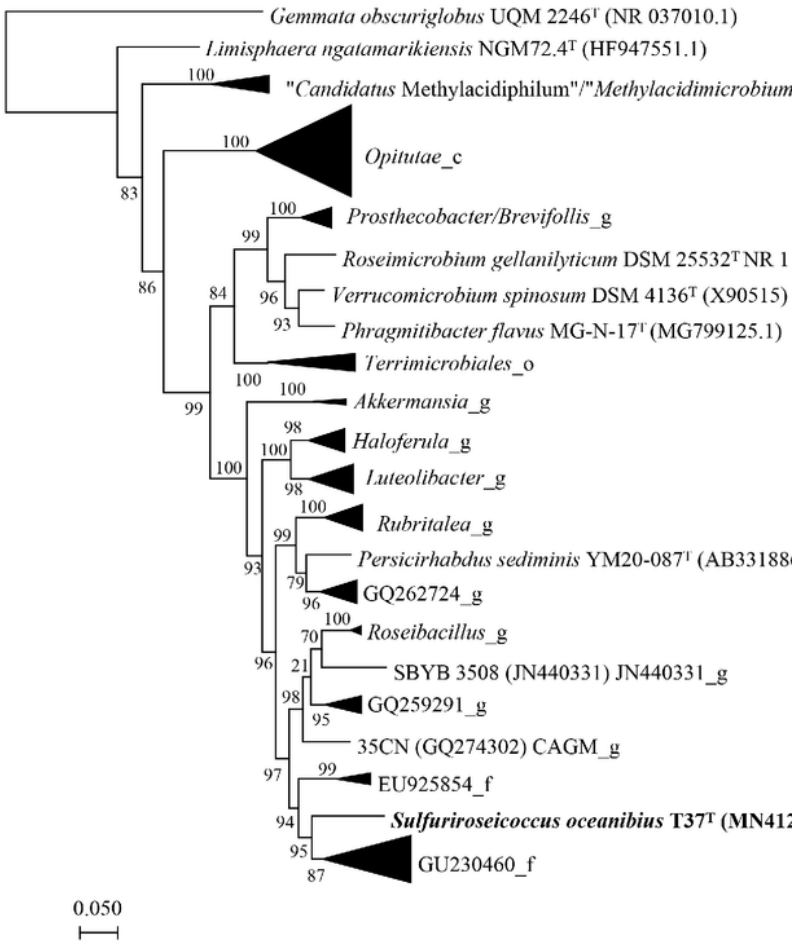

Figure 2

Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of strain T37T among closely related taxa. Gemmata obscuriglobus UQM 2246T (NR_037010.1) was used as an outgroup. Respective GenBank accession numbers for 16S rRNA genes are indicated in parentheses. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate a majority consensus tree. Bar, 0.02 nucleotide substitutions per position.

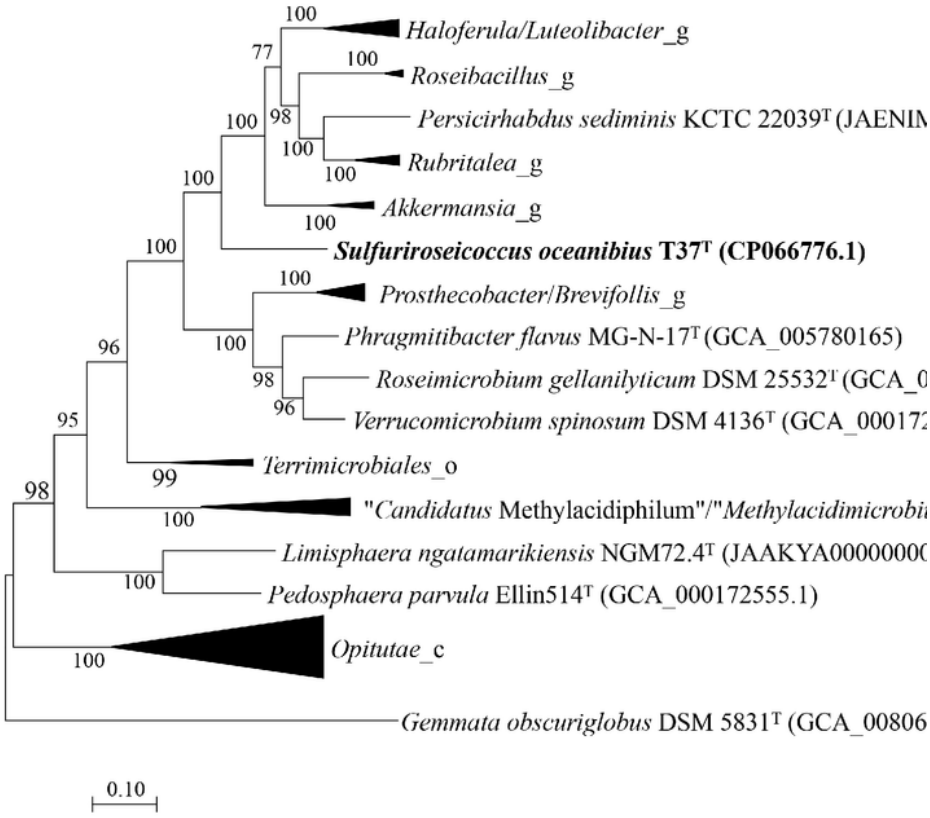

Figure 3

Phylogenetic analysis of the genomic sequences of performed with the IQtree showing the phylogenetic position of strain T37T among closely related taxa. G. obscuriglobus UQM 2246T was used as an outgroup.

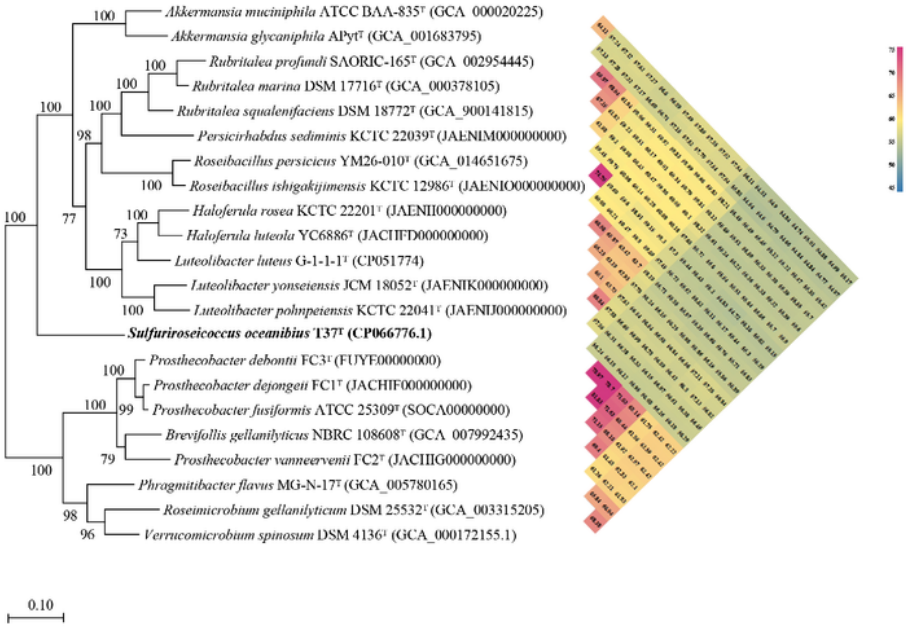

Figure 4

Heat map generated with AAI values between strain T37T and other closely related species with standing in nomenclature.

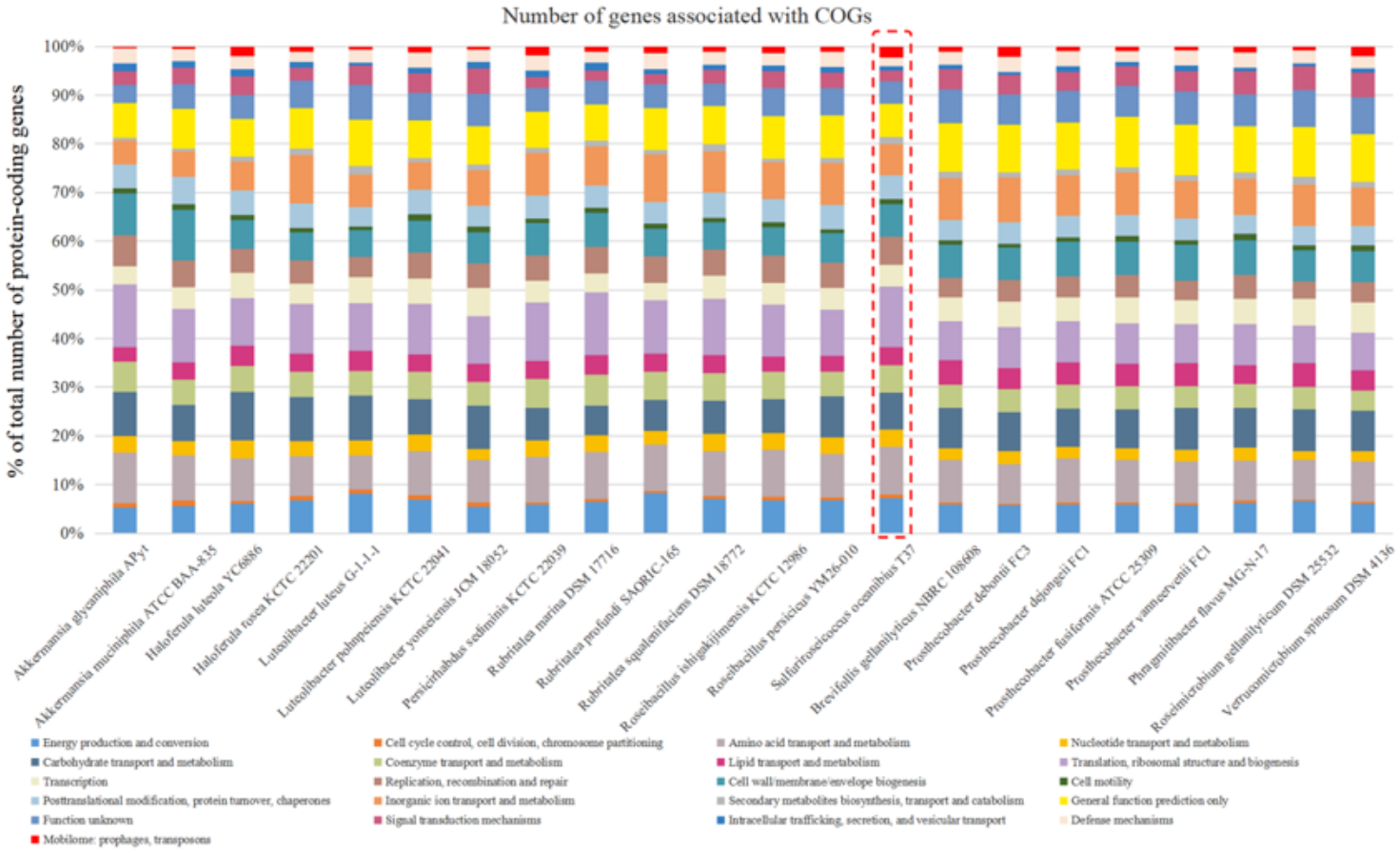

Figure 5

Comparison of the proportion of genes associated with the COGs functional categories of strain T37T and other closely related species.

Supplementary Files

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- SupplementaryMaterial.docx