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Brevundimonas fontaquae sp. nov., isolated from Dalgi carbonate spring-water

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달기 약수 지하수에서 분리한 신종 세균 *Brevundimonas fontaquae* sp. nov.

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Brevundimonas sp. CS1^T, isolated from Dalgi carbonate spring water in Cheongsong-up, Korea, is Gram-negative, aerobic, motile, and rod-shaped. Cells (0.5 µm × 1.2–2.6 µm) contain a single polar flagellum. The isolated colonies were round, viscous, and an orange color when incubated for 3 days at 28°C on nutrient agar. Furthermore, the strain (CS1^T) produces a diffusible brown pigment when grown on tryptic soy broth agar. CS1^T was positive for oxidase, DNase, and catalase activities, and positive for Tween 20, carboxymethyl cellulose, and starch hydrolysis. CS1^T was further characterized using API 20 NE, API 50CH, and API ZYM kits. The major fatty acids included $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c* (47.31%) and $C_{16:0}$ (17.61%). Non-phosphorylated glycolipids were the dominant polar lipids. Coenzyme Q-10 was predominantly detected as a quinone in this strain. The CS1^T genome was sequenced using Illumina and Oxford nanopore sequencing platform. The genome size was 3.3 Mb with a G + C content of 66.1 mol%. No plasmids, CRISPR array genes, or complete prophages were detected. Interestingly, the genome encodes P-type conjugative transfer proteins. Average nucleotide identity (ANI) value between CS1^T and the most closely related type strain, *B. vesicularis* NBRC 12165^T, was 94.27%. Comparison of CS1^T and strains to closely related type species based on polyphasic taxonomic analysis, ecology, and genome-based phylogeny indicated strain CS1^T is a novel species. Thus, we propose the name *Brevundimonas fontaquae* for CS1^T, whose strain type is CS1^T (= KCTC 82559^T = JCM 34943^T).

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The bacterial genus, *Brevundimonas*, belonging to the family *Caulobacteraceae*, was first proposed by reclassification of two strains belonging to the genus *Pseudomonas*, *P. diminuta*, and *P. vesiculare* (Segers *et al.*, 1994). The etymology of *Brevundimonas* is derived from a bacterium with a short-wavelength flagellum. Members of the genus *Brevundimonas* are Gram-negative rods that are motile with a polar flagellum, aerobic or facultative anaerobic, and non-fermentative. They have high DNA G + C contents (65–68%), $C_{16:0}$ and $C_{18:1}$ as major fatty acids and $C_{12:0}$ 3OH as a characteristic fatty acid, Q-10 as the predominant ubiquinone, and glycolipids as the main components of polar lipids (Ballard *et al.*, 1968; Wilkinson and Galbraith, 1979; Segers *et al.*, 1994). Members of *Brevundimonas* inhabit diverse environments, including soils and aquatic environments, and are related to humans, even as opportunistic pathogens (Ryan and Pembroke, 2018; Son *et al.*, 2022b). According to the LPSN (List of Prokaryotic names with Standing in Nomenclature), 34 species with the validly published names were currently listed in the genus *Brevundimonas* (https://lpsn.dsmz.de/ accessed on December 12, 2022). A recent investigation based on genome sequences in the database suggested the presence of 29 unclassified novel

species or corrections for species assignment in *Brevundimonas* (Liu *et al.*, 2021).

Specifically, *B. vesicularis* NBRC 12165^T was isolated from the urinary bladder epithelium of the leech, *Hirudo medicinalis* (Segers *et al.*, 1994). *Brevundimonas vesicularis* has been mainly identified from bacteremia or sepsis in patients with immunosuppression or in neonates (Ryan and Pembroke, 2018). In some cases, the strains of this species have been implicated in urinary tract, endocarditis, and cutaneous infections. Approximately 70% of the *Brevundimonas* infection cases are caused by *B. vesicularis* (Ryan and Pembroke, 2018). *Brevundimonas vesicularis* was also isolated from a paper machine (Verhoef *et al.*, 2002), freshwater (Beilstein and Dreiseikelmann, 2006; Nicoletti *et al.*, 2015), a biofilm reactor for 2,4-D treatment (Dargahi *et al.*, 2021), and from soil as rhizobacteria (Moon and Ali, 2022). These strains in the studies were identified based on 16S rRNA gene sequence and phenotype similarities, hence it is necessary to determine the phylogenetic positions of the strains with generally acceptable classification measures. In combination with distinctive phenotypic and chemotaxonomic characteristics, the widely accepted standards for delineation of bacterial species are based on the defined similarity values of the 16S rRNA gene or genomic sequences between type strains (Chun *et al.*, 2018). For example, compared to the closest phylogenetically related species, novel species have less than 98.7% of the 16S rRNA gene sequence similarity or less than 95–96% of ANI (average nucleotide identity) and 70% of dDDH (digital DNA-DNA hybridization) values, respectively.

The Dalgi (sometimes Dalki) spring waters at Cheongsong-up in Korea comprise of infiltrated carbonate water that rises along the fissure of the Jurassic granite in the valley floor of the Chenongson-up area, which contains carbon dioxide of magma origin (Jeong and Jeong, 1999). There are three main sites in a specific area: upper, middle, and lower spring water spots. In addition to HCO_3^-, this mineral water is rich in iron and other minerals, and is renown to Koreans as naturally carbonate water that is useful for 'gastric relief'. For instance, the spring water flow at the middle spring water site is 15 ml/min, with a pH of 6.17, > 1000 ppm CO_2, approximately 2.4 ppm dissolved oxygen, and Ca, Mg, Na, K, Si, Fe, and Mn ions (Lee, 2000). To date, bacterial isolation from these spring waters has not yet been reported. Here, we isolated a bacterial strain that predominately exists in the middle spring water site and determined its taxonomic position via polyphasic approaches, including genomic, phenotypic, and chemotaxonomic analyses.

Materials and Methods

Bacterial strain isolation

Water from a spring water site (36°26'23.7"N 129°05'14.2"E) in Cheongsong-up was collected in a sterile 50 ml falcon tube (Dec. 20, 2019) and stored in an ice bag. The following day, 50 µl was spread on nutrient agar (NA) and incubated for 3 days at 28°C. Orange colonies were observed, and a single colony, named CS1^T, was purely cultured via continuous streaking on NA and preserved in a 20% glycerol solution at -72°C.

16S rRNA gene sequence determination

The 16S rRNA gene was amplified by PCR (TRIO thermal cycler, Biometra) with primers 27F (5'-AGAGTTTGATCCT GGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for species identification, and the resulting nucleotide sequence obtained by Sanger sequencing method (Genotech Co.) was used for homology analysis in the NCBI database. The amplification conditions were the same as those previously described (Son *et al.*, 2022a).

Genome sequencing and genomic analyses

Cells were cultured in nutrient broth (NB) medium for 48 h at 28°C with shaking at 140 rpm for DNA extraction. Total genomic DNA was purified using the phenol extraction method (Ausubel *et al.*, 1988). Genomic DNA was then sequenced using both Illumina and Oxford Nanopore platforms. Illumina sequencing was performed on the Illumina NovaSeq6000 sequencer at DNALink Co. using a TruSeq DNA PCR-free 550 bp library kit (Illumina), as described previously (Son *et al.*, 2022b). The mean Phred Quality Score obtained was 35.48. For Nanopore sequencing, libraries were prepared using the SQK-LSK109 kit (Nanopore) and multiplexed using the EXP-NBD104 barcoding kit (Nanopore) according to the manufacturer's protocol. Sequencing was performed on a MinION sequencer using a FLO-MIN106 flow cell (r9.4.1) and

MinKNOW v20.10.3 software with default settings. The reads were assembled *de novo* using Unicycler (SPAdes v3.12.0) with the default settings (Wick *et al.*, 2017). The quality of the assembled genome sequences was evaluated using CheckM v1.1.3 (Parks *et al.*, 2015). Gene predictions and annotations were provided by NCBI using the best-placed reference protein set GeneMarkS-2+ of the NCBI Prokaryotic Genome Annotation Pipeline v5.1 (Tatusova *et al.*, 2016). The protein sequence-based comparison between genomes was performed using Rapid Annotations using Subsystems Technology (RAST) Seed Viewer (https://rast.nmpdr.org/) (Aziz *et al.*, 2008). The cumulative GC skew and GC content were depicted using the CGview program with sliding window size of 1,000 bp and 100 bp steps (https://proksee.ca/) (Grant and Stothard, 2008). The prophage genes in the genome server were screened using the PHASTER program (https://phaster.ca/) (Zhou *et al.*, 2011).

The genome-based taxonomy analysis was conducted using type (strain) genome serve (TYGS) (https://tygs.dsmz.de; [Meier-Kolthoff and Goker, 2019]). The phylogenetic tree was constructed using FastME 2.1.6.1 (Lefort *et al.*, 2015) from the genome BLAST distance phylogeny (GBDP) distances (formula d_5). dDDH values and confidence intervals were calculated using the recommended settings of the Genome-to-Genome Distance Calculator (GGDC 2.1). The type-based species clustering using a 70% dDDH radius around each of the 13 type strains was performed. Branch support was inferred from 100 pseudobootstrap replicates each. Default parameters were used for all software, unless noted otherwise. The results were calculated by the TYGS on Nov. 07, 2022. The average nucleotide identity (ANI) values between strain CS1^T and closely related type strains in the reference database GenomesDB were initially searched using the pairwise genome comparison service of JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/) (Richter *et al.*, 2016). Finally, the relationship between the ANI values of strain CS1^T and selected type strains was evaluated using orthoANI calculator (https://www.ezbiocloud.net/tools/ani) (Yoon *et al.*, 2017).

Phenotypic, biochemical, and chemotaxonomic characterization

Gram staining was performed using cells from the exponentially growth phase cultured in NB medium (Gerhardt, 1994), and motility was observed using phase-contrast microscopy (Nikon). Cellular morphology and flagella were observed using transmission electron microscopy (TEM) (Libra 120, Carl Zeiss), with phosphotungstic acid as a negative stain. Cell motility was also determined using agar stabs and inoculating cells into plates with NB medium containing 0.3% (w/v) agar followed by incubation at 28°C for 3 days. Anaerobic respiration was evaluated by growth on a fluid thioglycolate medium, which is used to assess the bacterial oxygen requirements for respiration. Another experiment evaluated cell growth on NB medium in a closed jar with an anaeroBag (Chongqing Pang Tong Medical Devices Co.) for 10 days at 28°C.

The temperature range for cell growth on NB medium was initially set at 5, 10, 15, 20, 25, 28, 30, 37, 40, and 45°C for 3 days. Subsequent experiments were performed by narrowing the temperature range. The pH range for growth on NB medium was tested from pH 3.0 to pH 13.0 (at 1 pH unit intervals) at 28°C for 3 days. The medium pH was adjusted after autoclaving at 121°C for 15 min using 1 N NaOH and 1 M HCl. The range of NaCl tolerance for growth on NB medium was tested from 0 to 10% (w/v) NaCl (at 1%-unit intervals) at 28°C for 3 days.

The following biochemical tests and physiological analyses of CS1^T, were conducted using cells grown on NB or on the indicated medium for 3 days at 28°C. All tests were conducted using protocols provided by the American Society of Microbiology (https://asm.org/Browse-By-Content-Type/Protocols) or were techniques in general use (Gerhardt, 1994). Control bacterial strains showing negative and positive activities in each test were simultaneously included. DNase activity was tested by growing the cells on DNase agar with methyl green (Kisan Bio). Coagulase activity was tested using rabbit plasma fibrinogen (Kisan Bio) followed by incubation at 35°C for 4 h to examine clot formation. Haemolytic activity was tested on blood agar media with sheep blood defibrinated (Kisan Bio) at 35°C for 20 h. Hydrolysis of carboxymethyl cellulose (1.0%, w/v), starch (0.2%, w/v), skimmed milk (1.4%, w/v), and Tween 20 (1.0%, w/v) was assessed on NA. To test the metabolism of various carbon sources and other biochemical characteristics of strain CS1^T, API 20 NE, and API 50 CH strips were used, according to the manufacturer's instructions (bioMérieux). For comparative analysis, *B. vesicularis* NBRC

12165^T was included in the tests. An API ZYM strip kit was used to test enzyme activities.

Fatty acid methyl esters were prepared according to the method described for the Sherlock Microbial Identification System (MIDI). Fatty acids were analysed using gas chromatography (GC) (Agilent 7890 GC-FID system) and identified utilising the Microbial Identification software package (v. 6.3), based on the Sherlock Aerobic Bacterial Database (TSBA60) (Sasser, 1990). Isoprenoid quinones of strain CS1^T were extracted, purified, and analysed using a reversed-phase HPLC system with a Fortis C18 column (5 µm, 4.6 × 150 mm) as previously described (Hiraishi *et al.*, 1996). Analyses of the composition of cellular fatty acids and quinones were carried out at AceEMzyme Co. Extraction and analysis of polar lipids were performed after cultivation in tryptic soy broth (TSB) medium at 28°C (approximately 400 rpm) for 3 days according to the method of Minnikin *et al.* (1984). Two-dimensional thin-layer chromatography (TLC) was performed using TLC Kiesel gel 60 (Merck) plates (10 × 10 cm). Polar lipids were first developed using chloroform/methanol/water (65:25:4, by volume), followed by chloroform/acetic acid/methanol/water (80:15:12:4, by volume). For colour development of total polar lipids, amino lipids, glycolipids, and phospholipids, sprays of ethanolic molybdophosphoric acid, ninhydrin, 1-naphthol-sulphuric acid, and molybdenum blue solutions were used, respectively (Da Costa *et al.*, 2011).

Scanning electron microscopy (SEM) and element analysis of crystalline particles

The shape and elemental composition of crystalline particles obtained from spring water precipitates were characterized by high-resolution field emission scanning electron microscopy (HR-FESEM, JSM-7900F/15 kV, JEOL) with an energy dispersive spectrometry (EDS) detector. The acceleration voltage was set to 3.00 kV.

Results and Discussion

Characterization of orange-colored precipitants from spring water by SEM-EDS

At the time of sample collection, orange precipitates formed after overnight storage. The particles were dried at 80°C and observed using FE-SEM to investigate whether microorganisms were attached to them. The particles were minute, less than 0.1 mm^2 in size with some particles that consisted of spikes, as shown in Fig. 1. EDS analysis revealed that the particles were composed of Ca, Mg, Si, and Fe at a ratio of 1:4.5:2.3:7.4 (atomic %). However, N and P were not detected, suggesting that there was no microbial origin. Hence, these particles were likely ejected from the underground springs and then slowly settled because of their small sizes and less dense morphology, as observed by SEM.

Growth and morphological characteristics of CS1^T

The isolated strain, CS1^T, grew on NB medium at 10–37°C (optimum, 28–32°C), at a pH of 6–10, and at 0–4% (optimum, 0–2%) NaCl concentrations. CS1^T also grew on Luria Bertani, Brain heart infusion, TSB, and Reasoner's (R2A) media. A brown diffusible pigment formed on TSB agar. The colonies

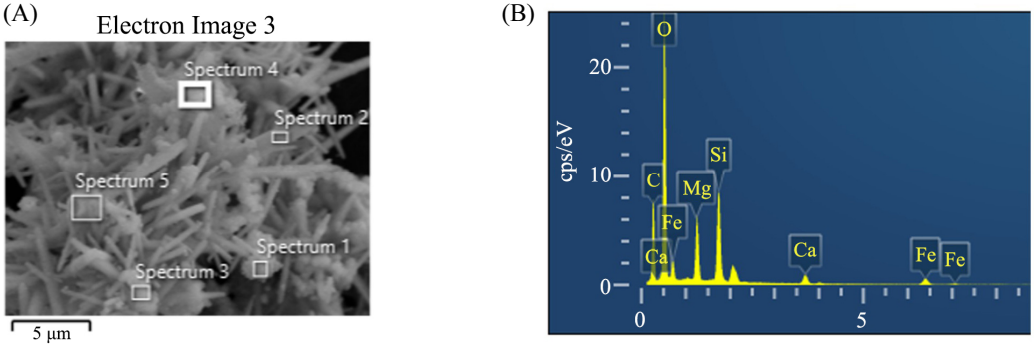

Fig. 1. Scanning electron microscopy (SEM) micrograph and energy dispersive spectrometry (EDS) analysis of microcrystalline particles settled down from spring water. (A) SEM image, (B) EDS result from spectrum 4 in box of (A). Note the elements of C and O were from the carbon tape and air, respectively.

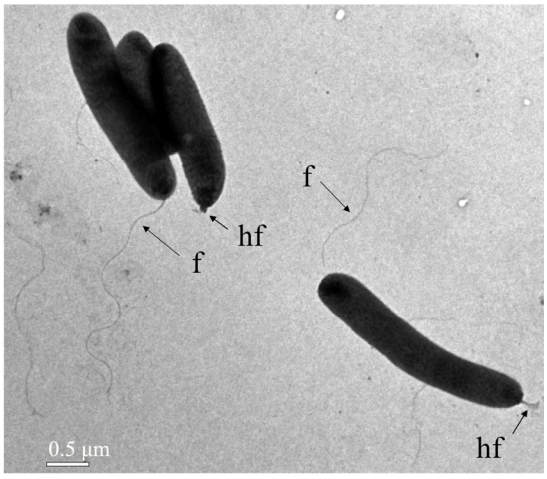

Fig. 2. Scanning electron microscopy (SEM) micrograph of cells of strain CS1^T. f, flagellum; hf, holdfast.

were round (2−3 mm diameter), viscous, and orange color when incubated for 3 days at 28°C. CS1^T did not grow under anaerobic conditions. The cells were rod shaped and motile. Cell size was 0.5 μm × 1.2−2.6 μm with a polar flagellum of 0.8 −1.2 μm wavelength (Fig. 2). Some cells contained a holdfast.

CS1^T identification via 16S rRNA gene sequence homology

The nucleotide sequence of the 16S rRNA gene (1,313 bp, GenBank accession number OP782576) was determined by PCR and Sanger sequencing. This sequence showed the highest homology with *B. vesicularis* NBRC 12165 (100.0%) and *B. nasdae* JCM 11415^T (100%). These strains were isolated from leeches, and the Russian Space Laboratory Mir, respectively (Segers *et al.*, 1994; Li *et al.*, 2004). In addition, other type strains showing more than 98.7% sequence similarity, which is the value used for species delineation (Chun *et al.*, 2018), were *B. intermedia* ATCC 15262^T (99.85%), *B. huaxiensis* 090558^T (99.6%), *B. mediterranea* DSM 14878^T (99.24%) and *B. aurantiaca* DSM 4731^T (99.09%). These were isolated from seawater, freshwater or human blood samples (Abraham *et al.*, 1999; Fritz *et al.*, 2005; Liu *et al.*, 2021). Since CS1^T has a different niche, we were interested in identifying the taxonomic characteristics of CS1^T and genome analyses.

General CS1^T genome features

Illumina sequencing yielded a total read length of 5.2 Gb with 1,718,516 reads. Nanopore sequencing yielded 145.1 Mb with 4,905 reads. The assembled genome with 1596.6× genome coverage yielded one circular contig of 3,300,602 bp (66.10% G + C content) with 3,164 protein-coding sequences, 48 t-RNA genes, four non-coding RNA genes, and two r-RNA gene operons. CheckM analysis revealed 99.68% estimated completeness and 0.97% estimated contamination. Of the protein coding genes, 62.7% had predicted functions and 28% were classified into protein subsystems by the PATRIC platform. For comparison, the genome of *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T possessed 3,358 kb and 3,164 kb, respectively. The G + C contents of these genomes were 66.3%, and 66.2%, respectively. In genome-based comparisons, CS1^T showed the highest ANI values of 94.27% (57.0% average reciprocal genome coverage) and 93.55% (56.6%) to *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T, respectively. The dDDH values in the same comparison were 56.4% and 53.2%, respectively. CS1^T also showed next high ANI values to *B. mediterranea* DSM 14878^T, *B. aurantiaca* DSM 4731^T, and *B. nasdae* JCM 11415T^T with 84.59%, 83.59%, and 82.91%, respectively. The dDDH values were 27.4%, 26.4%, and 26.0%, respectively. Genome information for *B. intermedia* ATCC 15262^T is not available.

Whole gene sequence-based CS1^T phylogeny

In genome-based taxonomy analysis using type (strain) genomes, CS1^T is classified as a phylotype with *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T but recognized as a separate species (Fig. 3). In the subtree, CS1^T and *B. vesicularis* NBRC 12165^T formed a separate group, with each species having similar distance to *B. huaxiensis* 090558^T. In contrast the close similarity of the 16S rRNA gene sequence, *B. nasdae* JCM 11415T^T, *B. aurantiaca* DSM 4731^T and *B. mediterranea* DSM 14878^T were more distantly related to CS1^T compared to *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T. In addition to the phylogeny, the ANI (94.27%) and dDDH (56.4%) values between CS1^T and *B. vesicularis* NBRC 12165^T were similar to the ANI (93.80%) and dDDH (53.9%) values between *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T. The calculated ANI and dDDH values between the three strains are below species-defining values (ANI > 95%, dDDH > 70%).

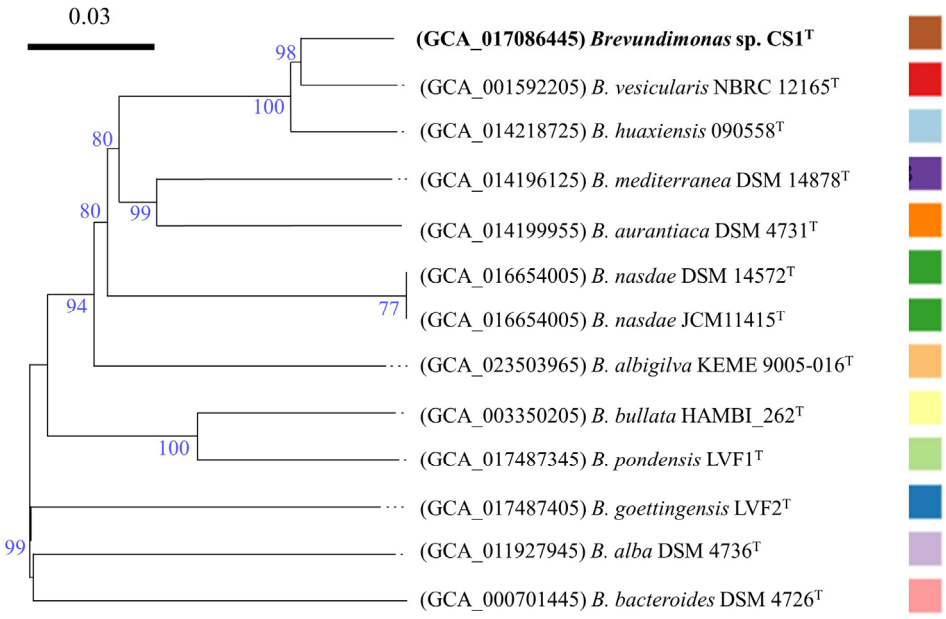

Fig. 3. Phylogenomic FastME tree showing the position of strain CS1^T. The branch lengths are scaled in terms of genome BLAST distance phylogeny (GBDP) distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications, with an average branch support of 89.3%. Bar, 3% nucleotide substitutions. GenBank assembly accession numbers are in a parenthesis. The color code presents species cluster.

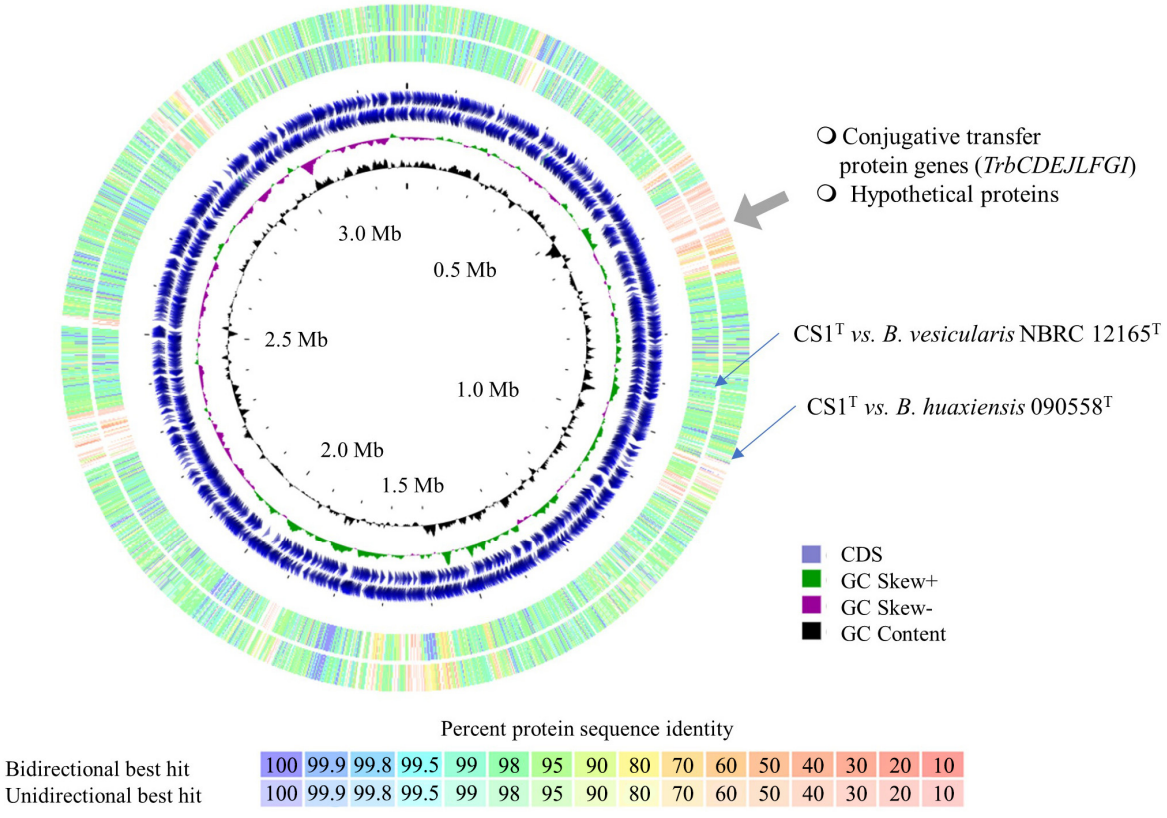

Fig. 4. Genome comparison of strain CS1^T to closely related strains conducted using Rapid Annotations using Subsystems Technology (RAST) (outside two tracks) and GC skew and GC content plots of strain CS1^T (inside). The tracks of strain CS1^T represents pairwise BLAST comparison between the ORFs in CS1^T genome against those in other strains, with percentages of identity represented with different colors shown in the legend.

Gene comparison of CS1^T to *B. vesicularis* NBRC 12165^T

Since CS1^T is most close to *B. vesicularis* NBRC 12165^T, we further compared the wo strains at the translated genes. In the RAST analysis, the number of coding sequences in CS1^T was 3,234 and, of which 383 genes were unique to CS1^T compared with those of *B. vesicularis* NBRC 12165^T. Unique genes have been annotated mostly to hypothetical proteins (278 genes), catabolism, the type II bacterial general secretion pathway, mobile elements, transporters, and transcriptional regulator functions. In addition, 282 genes were identified to have amino acid sequences identical to those of *B. vesicularis* NBRC 12165^T. Figure 4 shows the tracks of pairwise BLAST comparisons between the open reading frames (ORFs) in the CS1^T genome and those in *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T, with accompanying percentages of identity. Unusually, in the genomic tracks, gene clusters in CS1^T show lower genetic sequence homology than those in the other two species in several places. These regions were presumed to be gene clusters acquired via a horizontal (lateral) gene transfer. An example is a gene cluster including JX001_03290–JX001_033250 (*trbCDEJLFGI*) and *trbB* (JX001_03250), which code for P-type conjugative transfer proteins (Fig. 4) (Juhas *et al.*, 2008). Many genes in this gene cluster are hypothetical proteins. Since no plasmids were identified in CS1^T, the conjugative transfer proteins are presumed to be involved in the conjugal transfer of other mobile genetic elements, such as transposons. Genes encoding pseudopilin for the type II secretion system (*gspK* and *gspJIHG*) have also been identified in the genome. The CRISPR_array was not found in the CS1^T genome, and one gene cluster (JX001_1471–JX001_14790) encoding a gene transfer agent (GTA), involved in phage-like gene transfer, was found. Intact prophage genes were not identified in the Phaster analysis. A previous study also did not identified CRISPR-cas-related genes or plasmid replicons in the genomes of 24 species of *Brevundimonas* (Huang *et al.*, 2022). Therefore, since no studies on gene transfer and recombination have been conducted in *Brevundimonas* in detail, research in this field will prove interesting.

Phenotypic and chemotaxonomic characterization of CS1^T

The phenotypic properties of CS1^T were further characterized, as shown in Table 1. Characterization tests were conducted in parallel with *B. vesicularis* NBRC 12165^T. Table 1 shows the comparative results of different characteristics among *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T. The ability to hydrolyze Tween 20, produce a brown diffusible piment on TSB agar, assimilate D-trehalose and gentiobiose, and positive activity for lipase (C14), crystine arylamidase, 2-naphthyl-β-D-galactopyranoside (β-galactosidase), and β-glucosidase can differentiate CS1^T from the closest related strain, *B. vesicularis* NBRC 12165^T. *Brevundimonas vesicularis* NBRC 12165^T is differentiated from CS1^T by malic acid, D-ribose, D-galactose, and 5-ketogluconate assimilation. Furthermore, Coenzyme Q10 is unique isoprenoid quinone detected from CS1^T, the same as the other two strains. The major cellular fatty acids of CS1^T (> 5% of the total fatty acids) were $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c* (47.31%), $C_{16:0}$ (17.61%), and $C_{17:0}$ (6.60%), which showed similar patterns but different proportions compared with those of *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T. $C_{18:1}$ *ω5c* was identified uniquely in CS1^T (Table 2). On TLC for polar lipid analysis (Fig. 5), the spots designated for 1,2-di-O-acyl-3-O-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol (DGLs), 1,2-di-O-acyl-3-O-α-D-glucopyranosyl-sn-glycerol (MGD), and 1,2-di-O-acyl- 3-O-α-D-glucopyranuronosyl glycerol (MGDOx) were positive for 1-naphthol-sulfuric acid. The phosphatidylglycerol (PG) and phosphoglycolipid (PLG) spots were positive for molybdenum blue. Spots positive by ninhydrin reagent were not detected. The CS1^T polar lipid profile was similar to that of type strains of *Brevundimonas*, including *B. abyssalis* TAR-001^T (Tsubouchi *et al.*, 2013), *B. albigilva* NHI-13^T (Pham *et al.*, 2016), and *B. humi* CA-15^T (Dahal and Kim, 2018). Therefore, the chemical structures of the polar lipids of strain CS1^T were assigned according to those previous results. In addition, a similar pattern of polar lipids was obtained from *B. vesicularis* NBRC 12165^T (data not shown), of which the two-dimensional (2D) polar lipid pattern is unknown.

Taken together, compared to *B. vesicularis* NBRC 12165^T, strain CS1^T showed distinct phenotypic differences, especially when grown on TSB agar. CS1^T forms viscous, dark orange colonies that produce a brown pigment, whereas *B. vesicularis* NBRC 12165^T forms light orange colonies with a rough surface and no pigment production. These two strains were isolated

Table 1. Comparison of the phenotypic characteristics of CS1^T and closely related strains

	CS1^T	*B. vesicularis* NBRC 12165^T	*B. huaxiensis* 090558^T
Isolation	Carbonate-rich spring water	Leech, urinary-bladder	Human blood culture
Growth at 40°C	-	-	+
TSB agar	Dark orange, viscous colony surface, diffusible pigment	Pale orange, rough colony surface, no pigment	ND
Oxidase	+	+	-
Tween 20 hydrolysis	+	w	ND
API 20NE			
Nitrate reduction	-	-	+
Indole from L-tryptophan	-	-	+
Urease	-	-	+
β-Galactosidase*	-	-	+
Gelatin hydrolysis	-	-	+
Assimilation			
D-Glucose	+	+	-
N-Acetylglucosamine	-	w	+
D-Maltose	+	+	-
Potassium gluconate	-	-	+
Malic acid	-	+	-
API ZYM			
Lipase (C14)	+	-	-
Crystine arylamidase	+	-	-
Trypsin	+	+	-
α-Chymotrypsin	+	+	-
β-Galactosidase**	+	-	-
β-Glucosidase	+	-	-
API 50CH (assimilation)			
D-Trehalose	+	-	ND
Gentiobiose	+	-	ND
D-Ribose	-	+	ND
D-Galactose	-	+	ND
5-Ketogluconate	-	+	ND

Data for strains of CS1^T and *B. vesicularis* NBRC 12165^T are from this study, and *B. huaxiensis* 090558^T is from Liu *et al.* (2021). +, positive; -, negative; w, weak; ND, not tested. In the API 20NE test, all three strains were negative for arginine dihydrolase and hydrolyzed esculin. L-Arabinose, D-mannose, D-mannitol, capric acid, adipic acid, trisodium citrate, and phenylacetic acid were not assimilated. In the API ZYM test, all three strains were positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, lipase (C14), β-galactosidase, and were negative for α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. *Substrate is 4-nitrophenyl-β-D-galactopyranoside. **Substrate is 2-naphthyl- β-D-galactopyranoside.

from physiochemically distinct habitats. Along with their common characteristics, the two strains showed various differential phenotypic, biochemical, and chemotaxonomic characteristics. In addition, in genome-based comparisons, CS1^T in the phylogenetic tree is distantly related to its closest lineages, *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T, and the ANI and dDDH values of CS1^T to these strains are lower than those defining the species boundary. Consequently, strain CS1^T is proposed to be classified as a novel species separated from *B. vesicularis* NBRC 12165^T and *B. huaxiensis* 090558^T.

Description of *Brevundimonas fontaquae* sp. nov.

Brevundimonas fontaquae (font.a'quae. L. masc. n. *fons* spring; L. fem. n. *aqua* water; N.L. gen. n. *fontaquae* of spring water)

Cells are Gram-negative, aerobic, motile, and rod-shaped.

Table 2. Cellular fatty acid analysis of CS1^T and closely related strains

	CS1^T	*B. vesicularis* NBRC 12165^T	*B. huaxiensis* 090558 ^{T}
Saturated			
$C_{14:0}$	2.64	3.32	3.2
$C_{16:0}$	17.61	17.38	25.2
$C_{17:0}$	6.60	6.62	1.2
Unsaturated			
$C_{17:1}$ *ω6c*	4.95	6.87	1.1
$C_{17:1}$ *ω8c*	7.42	9.48	1.8
$C_{18:1}$ *ω5c*	1.38	ND	ND
Branched-chain fatty acid			
$C_{18:1}$ *ω7c* 11-methyl	3.46	4.12	2.6
Hydroxy fatty acids			
$C_{12:0}$ 3OH	2.21	3.19	2.1
Summed feature*			
3; $C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*	3.48	5.28	4.0
8; $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c*	47.31	40.95	55.9

Data for strains of CS1^T and *B. vesicularis* NBRC 12165^T are from this study and *B. huaxiensis* 090558^T from Liu *et al*. (2021).
In the total composition, only more than 1% of total cellular fatty acids are present. ND, not detected, Notes: minor fatty acids (< 1%) for CS1^T are $C_{9:0}$, $C_{12:0}$, $C_{13:0}$, $C_{18:0}$, $C_{19:0}$, $C_{15:1}$ *ω8c*, $C_{16:1}$ *ω5c*, $C_{20:4}$ *ω6*,9,12,15*c*, $C_{15:0}$ iso, $C_{11:0}$ 3OH, $C_{12:1}$ 3OH, summed features 1; $C_{15:1}$ iso H/$C_{13:0}$ 3OH and summed features 7; $C_{19:1}$ *ω6c*/ $C_{19:1}$ *ω7c*. *Summed features represent fatty acids that could not be separated using the MIDI system.

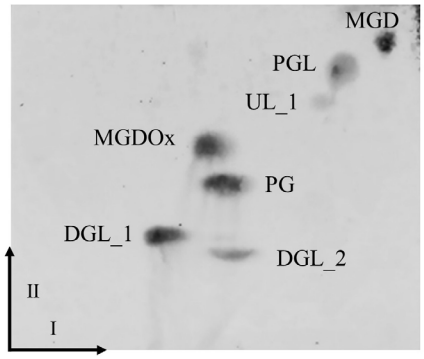

Fig. 5. Two-dimensional thin-layer chromatography (TLC) of polar lipids of CS1^T. The 5% ethanolic molybdophosphoric acid spray reagent was used to detect total lipids; Abbreviations: DGL, 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol; MGD, 1,2-di-*O*-acyl-3-*O*-[α-D-glucopyranosyl]-sn-glycerol; MGDOx, 1,2-di-*O*-acyl-3-*O*-α-D-glucopyranuronosyl glycerol; PGL, phosphoglycolipid; PG, phosphatidylglycerol; UL_1, unidentified non-phosphatidyl polar lipid.

Cells (0.5 μm × 1.2–2.6 μm) contain a single polar flagellum. The strain grows on nutrient broth at a temperature of 10–37°C (optimum, 28–32°C), at a pH of 6.0–10.0 and at 0–4% (optimum, 0–2%) NaCl concentration. The isolated colonies were round (2–3 mm diameter), viscous, and orange color when incubated for 3 days at 28°C on nutrient agar and produces a diffusible brown pigment when grown on tryptic soy broth agar. CS1^T is oxidase- and DNase-, catalase-positive, and coagulase- and hemolysin-negative, negative for casein (skimmed milk) hydrolysis, and positive for Tween 20, carboxymethylcellulose, and starch hydrolysis. In the API 20NE test, CS1^T was negative for nitrate reduction and indole production from L-tryptophan, arginine dihydrolase, urease, 4-nitrophenyl-β-D-galactopyranoside (β-galactosidase). CS1^T hydrolyzes esculin but not gelatin. D-Glucose and D-maltose were assimilated; however, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid were not assimilated in CS1^T. In the API ZYM test, CS1^T was positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, lipase (C14), β-galactosidase, and β-glucosidase, and was negative for α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. In API 50CH, of the 49 carbohydrates tested, acid was produced from L-arabinose, D-galactose, D-glucose, esculin (ferric citrate), D-cellobiose, D-maltose. Furthermore, D-cellobiose, amidon (starch), D-trehalose, and gentiobiose were assimilated. Major fatty acids were a summed feature of $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c* and $C_{16:0}$. Coenzyme Q-10 was predominantly detected as a quinone in this strain. Phosphatidylglycerol and phosphoglycolipid were detected as phospholipids in addition to major portions of non-phosphoglycolipids.

The type strain, CS1^T (= KCTC 82559^T = JCM 34943^T), was isolated from a carbonate spring water, Cheongsong-up, Korea (36°26'23.7"N 129°05'14.2"E). The genome consists of a single circular chromosome of size 3.3 Mb and DNA G + C content is 66.1 mol%.

Genome sequence accession numbers

The assembled genome sequence of strain CS1^T was deposited in NCBI GenBank under accession number CP070968.1. Raw sequence data used for assembly were deposited in GenBank under SRA accession numbers SRX10145201 (Illumina Nova seq) and SRX10145202 (MinIon seq). The 16S rRNA gene

sequence of strain CS1^T retrieved from genome was deposited to GenBank under accession number OQ029541.

적 요

한국 청송읍의 달기 탄산천수에서 분리한 *Brevundimonas* sp. CS1^{T}는 그람염색 음성이며, 호기적이며, 운동성을 갖는 막대형이다. 세포의 크기는 0.5 μm × 1.2~2.6 μm이며, 극성 단일 편모를 갖고 있다. 분리된 콜로니는 nutrient 한천 배지에서 28°C, 3일 동안 배양했을 때 둥글고, 점성이 있으며, 오렌지색으로 관찰되었다. Tryptic soy broth 한천 배지에서는 확산성이 있는 갈색 색소를 생성했다. CS1^{T}은 oxidase와 DNase, catalase 활성을 보이고, Tween 20, carboxymethyl cellulose 및 전분을 가수분해하였다. API 20 NE, API 50CH 및 API ZYM 키트를 사용하여 추가적으로 CS1^{T}의 특성을 조사하였다. 주요 지방산은 $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c* (47.31%)와 $C_{16:0}$ (17.61%)이고, 비인산화 당지질이 극성 지질의 대부분을 차지하였다. 코엔자임 Q-10이 퀴논으로 검출되었다. CS1^{T}의 게놈의 염기서열은 Illumina 및 Oxford nanopore 시퀀싱 플랫폼을 사용하여 결정하였다. 게놈 크기는 3.3 Mb이며, 66.1 mol%의 DNA G+C 함량을 포함하였다. 플라스미드, CRISPR array 유전자 및 완전한 프로파지를 암호화하는 유전자군은 검출되지 않았다. 흥미롭게도 게놈은 P형 접합 전달(conjugal transfer) 단백질 유전자를 암호화하고 있다. CS1^{T}과 가장 밀접하게 관련된 기준 균주인 *B. vesicularis* NBRC 12165^T 사이의 average nucleotide identity(ANI) 값은 94.27%이다. 다상 분류학적 분석, 생태학 및 게놈 기반 계통발생학을 기반으로 밀접하게 관련된 기준 균주와 비교한 결과, CS1^{T}는 신종으로 간주되었다. 따라서 CS1^{T}을 *Brevundimonas fontaquae*^{T}라는 이름을 제안하고자 한다. 본 균주에 대한 기준 균주는 CS1^T(=KCTC 82559^T = JCM 34943^T)이다.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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