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Granulosicoccaceae fam. nov., to Include *Granulosicoccus antarcticus* gen. nov., sp. nov., a Non-phototrophic, Obligately Aerobic Chemoheterotroph in the Order *Chromatiales*, Isolated from Antarctic Seawater

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Abstract A Gram-negative, motile by tuft flagella, obligately aerobic chemoorganoheterotrophic, sphere-form bacterium, designated IMCC3135^T, was isolated from the Antarctic surface seawater of King George Island, West Antarctica. The strain was mesophilic, neutrophilic, and requiring NaCl for growth, but neither halophilic nor halotolerant. The 16S rRNA gene sequence analysis indicated that the strain was most closely related to genera of the order *Chromatiales* in the class *Gammaproteobacteria*. The most closely related genera showed less than 90% 16S rRNA gene sequence similarity and included *Thioalkalispira* (89.9%), *Thioalkalivibrio* (88.0%–89.5%), *Ectothiorhodospira* (87.9%–89.3%), *Chromatium* (88.3%–88.9%), and *Lamprocystis* (87.7%–88.9%), which represent three different families of the order *Chromatiales*. Phylogenetic analyses showed that this Antarctic strain represented a distinct phylogenetic lineage in the order *Chromatiales* and could not be assigned to any of the defined families in the order. Phenotypic characteristics, including primarily non-phototrophic, non-alkaliphilic, non-halophilic, and obligately aerobic chemoheterotrophic properties, differentiated the strain from other related genera. The very low sequence similarities (<90%) and distant relationships between the strain and members of the order suggested that the strain merited classification as a novel genus within a novel family in the order *Chromatiales*. On the basis of these taxonomic traits, a novel genus and species is proposed, *Granulosicoccus antarcticus* gen. nov., sp. nov., in a new family *Granulosicoccaceae* fam. nov. Strain IMCC3135^T (=KCCM 42676^T=NBRC 102684^T) is the type strain of *Granulosicoccus antarcticus*.

Keywords: Family *Granulosicoccaceae*, *Granulosicoccus antarcticus*, marine bacterium, Antarctic, 16S rRNA gene

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The order *Chromatiales* [14] in the class *Gammaproteobacteria* encompasses physiologically diverse members of Gram-negative bacteria that include phototrophic purple sulfur bacteria and chemoautotrophic bacteria. Because of the alkaliphilic, halophilic, and photosynthetic characteristics of members of the order *Chromatiales*, several strains in the order have been consequently subjected to biotechnological purposes [3, 5, 10, 26]. The order *Chromatiales* currently contains three families, *Chromatiaceae* [15], *Ectothiorhodospiraceae* [15], and *Halothiobacillaceae* [21], which have been delineated largely based on the 16S rRNA gene phylogeny. The family *Chromatiaceae* currently contains 24 validly published genera, most of which show the typical characteristics of purple sulfur bacteria, including photolithoautotrophic growth with sulfide or elemental sulfur as electron donors and photoorganoheterotrophic growth with organic acids [14]. The family *Halothiobacillaceae* includes two non-phototrophic obligately chemolithotrophic genera: *Halothiobacillus* [22] and *Thiovirga* [18]. Among the families in the order *Chromatiales*, the family *Ectothiorhodospiraceae* shows the most versatile metabolisms, including extremely halophilic, extremely alkaliphilic, photolithotrophic, photoorganotrophic, and chemolithotrophic sulfur-, nitrite-, arsenite-oxidizing properties [15]. Only one genus, named *Arhodomonas*, among the 9 validly published genera in the family *Ectothiorhodospiraceae*, has been reported for an obligately aerobic chemoheterotrophic member [1].

Here, we report the identification and characterization of a novel obligately aerobic, non-phototrophic, non-alkaliphilic member of the order *Chromatiales*, isolated from Antarctic seawater. The phenotypic and phylogenetic characteristics of this bacterium, designated IMCC3135^T (IMCC represents the Inha Microbe Culture Collection), were significantly different from the characteristics of all the genera reported in the order *Chromatiales*. Strain IMCC3135^T could not be assigned to any of the defined

families in the order *Chromatiales*, and phylogenetic characteristics together with phenotypic properties justify its characterization as a new genus and species in a novel family, named *Granulosicoccaceae* fam. nov.

MATERIALS AND METHODS

Strain Isolation

A seawater sample was collected from a coast of the Weaver Peninsula, King George Island, Antarctica (62°14'S, 58°47'E), in December 2005. One hundred μl of the seawater sample was spread onto an oligotrophic medium, R2A agar (Difco) diluted in aged Antarctic seawater 1:10 (v/v, 1/10R2A), and the agar plates were incubated aerobically at 20°C for one month. Strain IMCC3135^T, initially grown on 1/10R2A, was further purified on marine agar 2216 (MA; Difco) after growing the strain at 20°C for one week. After its optimum growth temperature was determined, cultures of strain IMCC3135^T were maintained routinely on MA at 20°C and preserved as a glycerol suspension (10%, v/v) at −70°C.

Phenotypic Characterization

Cell morphology, including the presence of flagella and intracellular granules, was examined by transmission electron microscopy (CM200, Philips, The Netherlands) using a 4-day culture on MA at 20°C. Cell size was measured using phase-contrast and epifluorescence microscopy (Nikon 80i, Nikon, Japan). For electron microscopy, exponential phase cells were washed twice with sodium cacodylate buffer, and negatively stained with 2% phosphotungstic acid (pH 7.0–7.2) on Formvar-filmed copper grids. Colony morphology, size, and color were examined from cultures grown aerobically on MA for 3 days and 2 weeks. Anaerobic growth was tested using both the MGC anaerobic system and the AnaeroPACK·Anaero (Mitsubishi Gas Chemical company, Inc.) under light and dark culture conditions. Flagellar motility was examined using wet mount preparations, made from exponential-phase cells. Growth temperature range and optimum were tested at 3–42°C. The pH range and optimum for growth were examined at pH values of 4.0–12.0. The NaCl concentrations and optimum for growth were determined in NaCl-free artificial seawater medium (ASW; basic formula, l^{-1}: 5.9 g $MgCl_2 \cdot 6H_2O$, 3.24 g $MgSO_4 \cdot 7H_2O$, 1.8 g $CaCl_2 \cdot 2H_2O$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.08 g KBr, 0.034 g $SrCl_2 \cdot 6H_2O$, 0.022 g H_3BO_3, 0.008 g $Na_2H_2PO_4$, 0.004 g Na_2SiO_3, 0.0024 g NaF, 0.0016 g KN_4NO_3), supplemented with 5.0 g peptone, 1.0 g yeast extract, and various concentrations of NaCl (0–15%, w/v). The range of temperature, pH, and NaCl concentrations for growth was monitored for 2 weeks. The catalase test was performed by addition of 3.0% hydrogen peroxide to exponential-phase colonies, and oxidase activity was determined using and oxidase reagent (bioMérieux). Other biochemical tests and carbon source utilization tests were carried out in API 20NE and API ZYM strips (bioMérieux) and in GN2 microplates (BIOLOG), according to the manufacturer's instructions, as described elsewhere [4, 8, 35, 36], and inoculated with bacterial suspensions in ASW. Susceptibility to antimicrobial agents [tetracycline (30 μg), ampicillin (10 μg), kanamycin (30 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin G (10 μg), streptomycin (10 μg), vancomycin (30 μg), and rifampicin (50 μg)] was determined using the diffusion method [19].

Chemotaxonomic Characterization

For the determination of the DNA G+C content of strain IMCC3135^T, genomic DNA was extracted and purified using the Qiagen DNeasy tissue kit according to the manufacturer's instructions. The DNA G+C content of the strain was analyzed by using HPLC, with a Discovery C18 column (5 μm, 15 cm×4.6 mm, Supelco). Cellular fatty acid methyl esters were extracted and prepared from cultures grown on MA at 20°C for 5 days, and analyzed according to the MIDI Microbial Identification System by the Korean Culture Center of Microorganisms (KCCM). Isoprenoid quinone content was analyzed using reversed-phase HPLC analysis by the KCCM [23, 28].

16S rRNA Gene Sequencing and Phylogenetic Analysis

PCR amplification of the bacterial 16S rRNA gene was performed with the slightly modified universal bacterial primers 27F-B and 1492R [7, 24]. PCR products were purified using a Qiagen QIAquick PCR purification column and sequenced directly by the chain-termination method. Initially, an almost full-length sequence of the 16S rRNA gene was compared with sequences available in GenBank by BLAST network searches [2] and RDP-II [9], to determine its approximate phylogenetic affiliation. Sequences were aligned using the ARB software package [25] and 1,116 unambiguously aligned nucleotide positions were used for phylogenetic analyses in PAUP* version 4.0 beta 10 [34]. The 16S rRNA gene sequence similarity scores were calculated from distance matrices based on the Jukes-Cantor distance formula [20] in the ARB. Phylogenetic trees were inferred by three different algorithms: neighbor-joining with the Kimura 2-parameter model; maximum-parsimony with a heuristic search; and maximum likelihood with a heuristic search, tree bisection-reconnection (TBR)-branching, and a Ti/Tv ratio of 1.6572. Tree topologies from neighbor-joining and maximum-parsimony were evaluated by bootstrap analyses based on 1,000 resamplings.

Nucleotide Sequence Accession Number

The 16S rRNA gene sequence of strain IMCC3135^T has been deposited in GenBank under the accession number EF495228.

RESULTS AND DISCUSSION

Phylogeny: The Basis for a New Family Delineation

A total of 1,481 nucleotides of the 16S rRNA gene sequence were determined for strain IMCC3135^T. Results of preliminary BLAST network searches and ARB tree analyses showed that strain IMCC3135^T belonged to the order *Chromatiales* in the class *Gammaproteobacteria*, and was most closely related to the uncultured marine hatchery clone ULV6 (GenBank Accession Number DQ357707) with 96.8% 16S rRNA gene sequence similarity. The sequence similarity of strain IMCC3135^T to other validly published bacterial species in the *Chromatiales* was very low, with no validly described bacterial species showing more than 90% sequence similarity. Sequence comparisons indicated that the strain was most closely related to the genera *Thioalkalispira* (89.9% sequence similarity), *Thioalkalivibrio* (88.0%–89.5%), and *Ectothiorhodospira* (87.9%–89.3%) in the family *Ectothiorhodospiraceae*, and the genera *Chromatium* (88.3%–88.9%) and *Lamprocystis* (87.7%–88.9%) in the family *Chromatiaceae*. As shown in the phylogenetic tree (Fig. 1), strain IMCC3135^T and uncultured bacterial clones formed a unique phylogenetic clade that did not associate significantly with any of the known three families of the order *Chromatiales*. This clade appeared to be monophyletic in all the phylogenetic trees generated in this study, with strong bootstrap supports. The very low sequence similarities (<90%) and distant relationships between stain IMCC3135^T and the other three families in the order *Chromatiales* suggested that the strain represented a novel genus and species within a new family in the order *Chromatiales*.

Phenotypic Characteristics of the Antarctic Isolate

Strain IMCC3135^T was a Gram-negative (by Gram-staining and KOH test), motile coccoid, 0.8–2.2 (average 1.2) μm

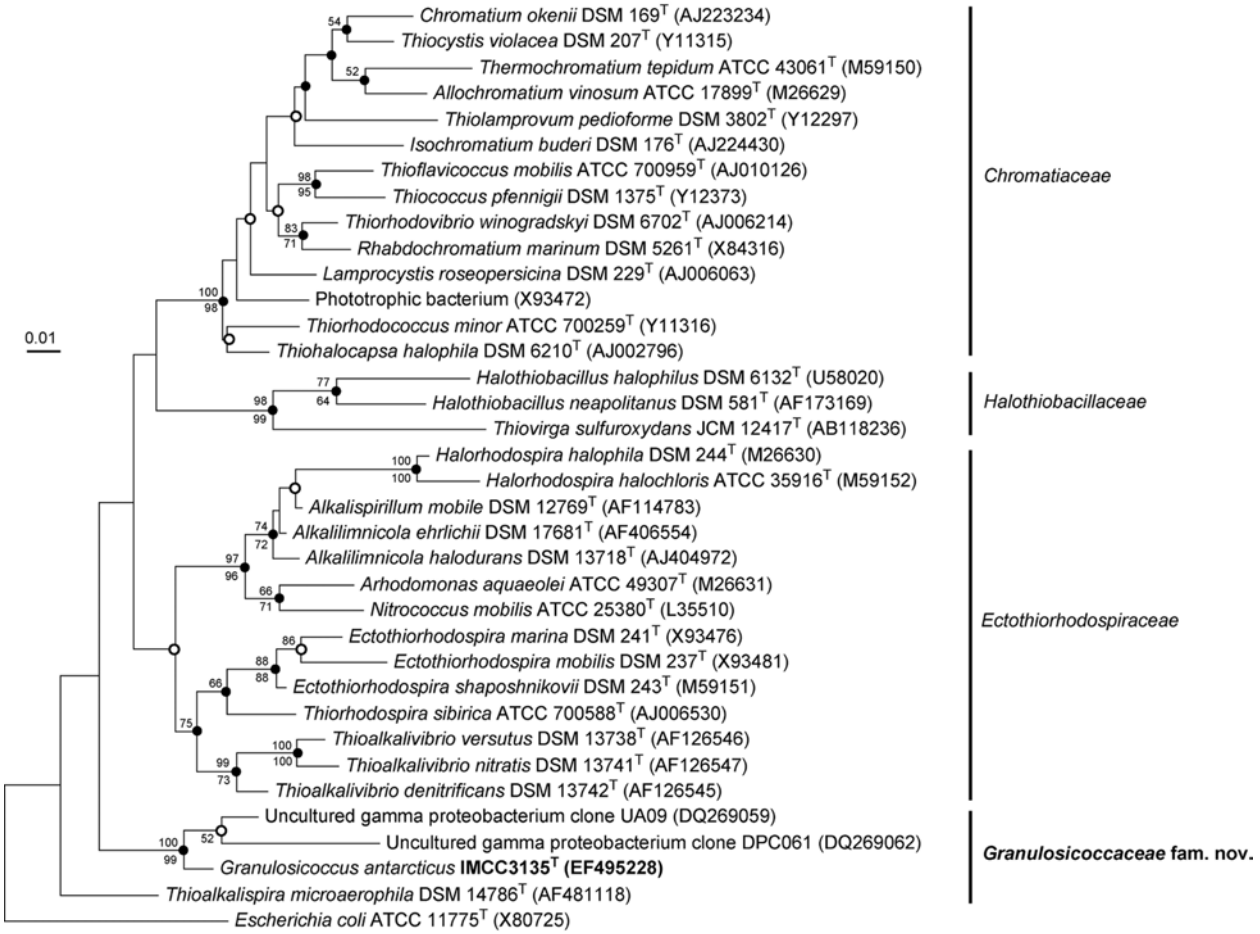

Fig. 1. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing distant relationships between strain IMCC3135^T and representatives of the order *Chromatiales*.

Bootstrap percentages (above 50%) from both neighbor-joining (above nodes) and maximum parsimony (below nodes) are shown. Filled and open circles at each node respectively indicate nodes recovered reproducibly by all treeing methods or by two treeing methods. Brackets indicate each family in the order *Chromatiales*. Bar, 0.01 substitutions per nucleotide position.

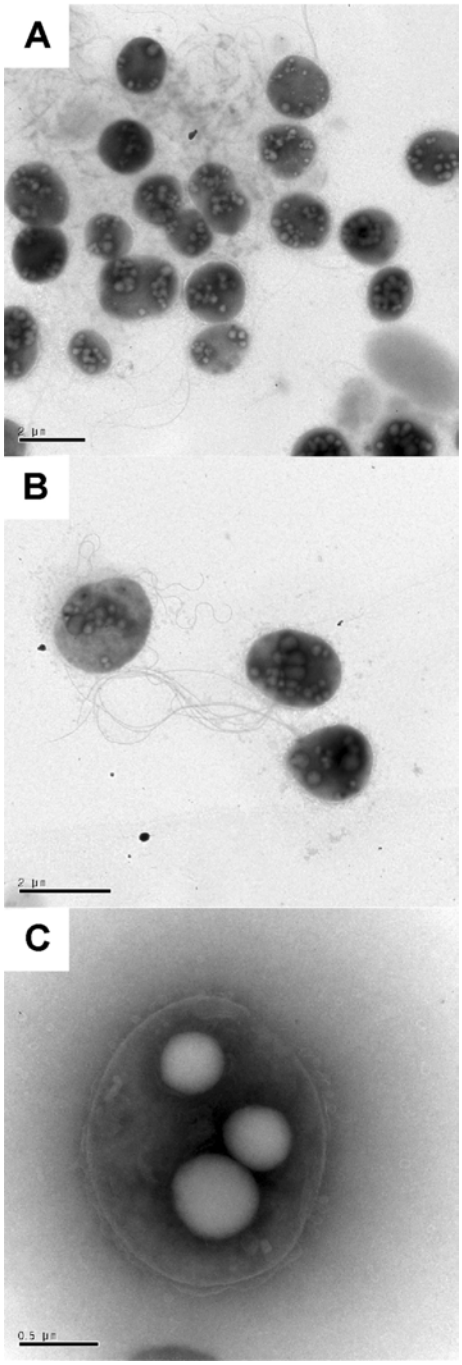

Fig. 2. Transmission electron micrographs of negatively stained cells of strain IMCC3135^T.
A. Coccoid cells showing a cell division by binary fission. **B**. Cells containing tuft flagella. **C**. Cells containing PHB granules.

in diameter, dividing by binary fission (Fig. 2A). The strain had long tuft flagella with a length of approximately 3–6 μm (Fig. 2B). The poly-β-hydroxybutyrate (PHB) granules were clearly visible in transmission electron micrographs (Fig. 2C) and Nile Blue A stained epifluorescence micrographs. Colonies of the strain grown on MA at 20°C for 3 days were 0.3–1.5 mm in diameter, beige to pale yellowish, circular, convex, viscous, opaque, and with entire margins. Colonies grown for 2 weeks formed star-shaped aggregates and the colony color changed to yellow. No growth was detected under anoxic conditions during the light (12 h/12 h:light/dark cycle) and dark incubation. Therefore, the strain was considered to be an obligately aerobic chemoheterotrophic bacterium. The temperature range for growth was 3–25°C, with optimum growth at 20°C. The strain grew optimally at pH 7.0 and 2.0% (w/v) NaCl. The strain oxidatively assimilated some hexoses, sugar acids, and amino acids, showing a typical property of chemoorganoheterotrophs. These basic metabolic properties differentiated the strain from members of the order *Chromatiales* (Table 1). Other physiological characteristics of the strain are fully described in the species description.

Chemotaxonomic Characteristics

In total, 12 different kinds of cellular fatty acids (each comprising >1%), containing 10–20 carbon atoms, were observed in strain IMCC3135^T (Table 2 and species description). The major cellular fatty acids detected in the strain were $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH (38.9%), $C_{18:1}$ ω7c (20.4 %), $C_{16:0}$ (13.2%), and $C_{10:0}$ 3-OH (9.5%), and the overall fatty acid composition was different from that of other species in the order *Chromatiales* (Table 2). The presence of $C_{10:0}$ 3-OH and relatively higher amount of $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH differentiated the strain from other distantly related species. The respiratory quinone detected was ubiquinone-8 (Q-8), differentiating the strain from the genera *Ectothiorhodospira* and *Halorhodospira* (Table 1). The DNA G+C content of strain IMCC3135^T was 58.0%, as determined by the HPLC method.

Taxonomic Conclusions

Even though strain IMCC3135^T was distantly related to the families *Chromatiaceae*, *Ectothiorhodospiraceae*, and *Halothiobacillaceae* in the phylogenetic trees, sequence similarities of the strain to other members of these families were only 85.8%–88.9%, 86.3%–89.9%, and 85.2%–88.4% respectively. Furthermore, a clear assignment of the strain to the known families in the order *Chromatiales* was not seen in all the phylogenetic trees generated by three treeing algorithms. From the phylogenetic analysis, the strain could not be associated with any of the three known families of the order *Chromatiales*; these novel strains therefore appear to constitute a new fourth family of the order. Strain IMCC3135^T was also physiologically differentiated from its closest neighbors, as shown in Table 1. Among the genera listed in Table 1, only the genus *Arhodomonas* shows the property of obligately aerobic chemoheterotrophic bacteria. Although the genus *Arhodomonas* is not phototrophic and obligately chemoorganoheterotrophic, the genus shows

Table 1. Differential characteristics of strain IMCC3135^T and other related genera in the order *Chromatiales*.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Morphology[a]	C	SR, LR	VI	LR	VI	VI, S	C	S	S	VB, S	SR	C	SR
Flagella[b]	PT	P	P	P	PT	P	P	P	P	PT	PT	P	P
PHB accumulation	+	V	ND	ND	+	+	+	–	–	–	+	–	–
Pigmentation[c]	–	–	–	–	R	R/G	–	Y	Y/B	R	PR	PR	Y
Temperature optimum (°C)	20	30–35	35–38	37	25–40	30–47	15–30	30	ND	25–30	20–35	23–27	28–42
pH optimum	7	9.3–9.5	9–10	7	7.6–10.0	8.0–9.2	6.8–8.0	10	9.0–10	9.0–9.5	7	7.0–7.6	6.5–8.0
NaCl range (%)	0.5–5.0	0–28	0–25	6–20	0–20	11–32	ND	2–9	2–23	0–6	ND	ND	23
NaCl optimum (%)	2.0	3–8	2	15	1–7	15–25	2–3	2.9	5–12	0.5–1	0	0–4	2.3–5.8
Aerobic growth	+	+	+	+	V	–	+	+	+	–	–	+	+
Anaerobic growth	-	+	+	–	+	+	–	–	V	+	+	+	–
Energy metabolism[d]	CH	CH, CA	CH,CA	CH	PA, PH, CA	PA. PH	CA	CA	CA	PA	PA, PH	PA, CA	CA
Bacteriochlorophyll	–	–	–	–	a	a, b	–	–	–	a	a	a	–
G+C content (mol%)	58.0	66–68	66.2	67	61–68	51–70	61.2	58.9	61–66	56–57	48–50	63–64	56
Quinone	Q8	ND	ND	ND	MK7/Q7/Q8	MK8/Q8	ND	ND	Q8	ND	ND	ND	Q8

Taxa: 1, Strain IMCC3135^T (present study); 2, *Alkalilimnicola* [11, 33, 39]; 3, *Alkalispirillum* [27, 33]; 4, *Arhodomonas* [1]; 5, *Ectothiorhodospira* [12]; 6, *Halorhodospira* [13]; 7, *Nitrococcus* [38]; 8, *Thioalkalispira* [31]; 9, *Thioalkalivibrio* [29, 30, 32]; 10, *Thiorhodospira* [6]; 11, *Lamprocystis* [16]; 12, *Chromatium* [17]; 13, *Halothiobacillus* [21]. Symbols: +, positive; –, negative; V, variable; ND, no data available.

[a]C, coccus; SR, short rod; LR, long rod; VI, vibrioid; S, spirilla.

[b]PT, polar or subpolar tuft flagella; P, 1 or 2 polar flagella.

[c]R, red; G, green; Y, yellow; B, brown; PR, purple red.

[d]CH, chemoheterotrophic; CA, chemolithoautotrophic; PA, photoautolithotrophic; PH, photoorganoheterotrophic.

an extremely halophilic property. This extreme halophilic property differentiated strain IMCC3135^T from the genus *Arhodomonas*. Strain IMCC3135^T was also differentiated from the other genera in the order *Chromatiales*, primarily in its non-alkaliphilic, non-phototrophic, and non-autolithotrophic properties.

It is evident from the low 16S rRNA gene sequence similarity (<90%), unique branching patterns in the phylogenetic analyses, and physiological characteristics that the Antarctic marine isolate cannot be assigned to any previously recognized bacterial family or genus and therefore should be characterized as a novel species within a new genus, *Granulosicoccus antarcticus* gen. nov., sp. nov., in a new family, *Granulosicoccaceae* fam. nov.

Table 2. Cellular fatty acid composition (%) of strain IMCC3135^T and related type strains of species in the order *Chromatiales*.

Fatty acid	1	2	3	4	5	6
$C_{10:0}$ 3-OH	9.5	–	–	–	–	–
$C_{12:0}$	2	–	6.4	–	–	–
$C_{14:0}$	–	4.1	–	–	1.2	0.3
$C_{16:0}$	13.2	23.4	15.4	21.7	35.7	10.8
$C_{16:1}$ ω9c	–	0.7	–	12.6[a]	2.7	0.6
$C_{16:1}$ ω9c/iso-$C_{15:0}$ 2-OH	38.9	–	–	–	–	–
$C_{17:0}$	–	1.1	–	–	–	–
$C_{18:0}$	–	12.1	6.8	–	7.6	11.7
$C_{18:1}$ ω7c	20.4	–	71.4	21.7[b]	–	–
$C_{18:1}$ ω7c/$C_{18:1}$ ω9c	–	54.0	–	–	16.5	62.8
$C_{19:0}$ d8,9	–	0.6	–	12.7[c]	33.8	5.8
$C_{20:1}$ ω9c	–	0.4	–	–	–	1.8
$C_{22:0}$	–	0.7	–	–	–	–

Strains: 1, Strain IMCC3135^T (present study); 2, *Alkalilimnicola halodurans* 34Alc^T [39]; 3, *Alkalispirillum mobile* SL-1^T [27]; 4, *Arhodomonas aquaeolei* HA-1^T [1]; 5, *Ectothiorhodospira mobilis* DSM 237^T [37]; 6, *Halorhodospira halophila* DSM 244^T [37].

[a]$C_{16:1}$; [b]$C_{18:1}$; [c]$C_{19:0}$.

Description of *Granulosicoccus* gen. nov.

Granulosicoccus (Gra.nu.lo.si.coc'cus. N.L. adj *granulosus* granular; N.L. masc. n. *coccus* coccus, a berry; N.L. masc. n. *Granulosicoccus* a granular coccus).

Cells are Gram-negative, strictly aerobic cocci that are motile by means of tuft flagella. NaCl is required for growth, but it is neither halophilic nor halotolerant. Obligately chemoorganoheterotrophic, mesophilic, and neutrophilic. Oxidase- and catalase-positive. The PHB granules are present as storage materials. The major fatty acids are $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH (38.9%), $C_{18:1}$ ω7c (20.4%), $C_{16:0}$ (13.2%), and $C_{10:0}$ 3-OH (9.5%). The only quinone detected is Q-8. The DNA G+C content of the type species is 58.0 mol%. Phylogenetically, the genus forms a novel fourth family of the order *Chromatiales* in the *Gammaproteobacteria*. The type species of the genus is *Granulosicoccus antarcticus*.

Description of *Granulosicoccus antarcticus* sp. nov.

Granulosicoccus antarcticus (ant.arc'ti.cus. L. masc. adj. *antarcticus* of the Antarctic environment, where the organism was isolated).

In addition to properties given in the genus description, the species is characterized as follows. Cell and colony

morphology and growth characteristics are given in the text. In API 20NE strips, positive for nitrate reduction, aesculin hydrolysis, gelatinase, and β-galactosidase activity; negative for indole production, acid production from glucose fermentation, arginine dihydrolase, and urease. In API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and α-chymotrypsin activities are present. Oxidatively assimilates the following carbon substrates in BIOLOG GN2 microplates: Tween 40, Tween 80, D-fructose, L-fucose, D-galactose, maltose, D-mannose, D-sorbitol, D-galacturonic acid, β-hydroxybutyric acid, α-ketoglutaric acid, malonic acid, succinamic acid, glucuronamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, L-serine, L-threonine, putrescine, 2-aminoethanol, and D,L-α-glycerol phosphate. Susceptible to chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin, and tetracycline. Resistant to ampicillin, penicillin G, and vancomycin. In addition to the major cellular fatty acids, the following fatty acids are presented in minor proportions: ECL 11.799 (2.6%), $C_{15:0}$ (2.2%), $C_{12:0}$ (2.1%), $C_{17:0}$ (1.6%), $C_{14:0}$ 2-OH (1.4%), $C_{14:0}$ (1.3%), $C_{10:0}$ (1.2%), and $C_{12:0}$ 2-OH (1.2%). Traces (<1%) of the following fatty acids are also present: ECL 18.846 and/or $C_{19:1}$ ω6c, $C_{17:1}$ ω7c, $C_{13:0}$ 2-OH, $C_{18:0}$, $C_{17:1}$ ω6c, $C_{13:0}$, $C_{11:0}$, and $C_{11:0}$ 3-OH. The type strain is IMCC3135^T (=KCCM 42676^T=NBRC 102684^T), isolated from the surface seawater of Maxwell Bay, King George Island, West Antarctica.

Description of *Granulosicoccaceae* fam. nov.

Granulosicoccaceae (Gra.nu.lo.si.co.cca'ce.ae. N.L. masc. n. *Granulosicoccus* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Granulosicoccaceae* the family of the genus *Granulosicoccus*).

The family *Granulosicoccaceae* encompasses obligately chemoheterotrophic Gram-negative bacteria within the order *Chromatiales* in the class *Gammaproteobacteria.* Currently, the family comprises the genus *Granulosicoccus*. The delineation of the family is primarily determined by the phylogenetic position of 16S rRNA gene sequences and the phenotypic properties of the genus *Granulosicoccus*. The detailed description is the same as for the genus *Granulosicoccus*. The type genus of the family is *Granulosicoccus.*

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