Granulosicoccus coccoides sp. nov., isolated from leaves of seagrass (*Zostera marina*)

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A non-pigmented, motile, Gram-negative bacterium, strain Z 271^T, was isolated from the surface of leaves of the seagrass *Zostera marina* which was collected in Troitza Bay (Sea of Japan, Pacific Ocean). The new isolate grew between 5 °C and 28 °C and was slightly halophilic, tolerating environments containing up to 5 % (w/v) NaCl. Strain Z 271^T was able to degrade Tweens 20, 40 and 80 and partially degrade gelatin, but was unable to degrade casein. Phosphatidylethanolamine (36.9 %) and phosphatidylglycerol (63.1 %) were the predominant phospholipids. The major fatty acids included C_{18:1}ω7c (43.7 %), C_{16:1}ω7c (31.1 %) and C_{16:0} (16.8 %). The main respiratory quinone was Q-8. The DNA–DNA relatedness value of strain Z 271^T with *Granulosicoccus antarcticus* IMCC3135^T was 35 %. The G + C content of the DNA of strain Z 271^T was 60.2 mol%. On the basis of phenotypic and genotypic characteristics and 16S rRNA gene sequence analysis, strain Z 271^T represents a novel species of the genus *Granulosicoccus* for which the name *Granulosicoccus coccoides* sp. nov. is proposed. The type strain is Z 271^T (=KMM 6014^T =CIP 109923^T).

The genus *Granulosicoccus*, with the single species *Granulosicoccus antarcticus*, was described in 2007 as a member of the newly proposed family *Granulosicoccaceae* (Lee *et al.*, 2007) of the order *Chromatiales* (Imhoff, 2005).

In this study, we report the characterization of a novel bacterium of the genus *Granulosicoccus*, isolated in July 1998 from leaves of the seagrass *Zostera marina*. The seagrass was collected in Troitza Bay of the Gulf of Peter the Great (Kurilenko *et al.*, 2007). A strain, Z 271^T, was isolated from an individual colony grown for 7 days at 28 °C on a medium (designated 'Medium A') containing: 0.2 % (w/v) Bacto peptone (Oxoid), 0.2 % (w/v) casein hydrolysate (Merck), 0.2 % (w/v) Bacto yeast extract

(Oxoid), 0.1 % (w/v) glucose, 0.02 % (w/v) KH₂PO₄, 0.005 % (w/v) MgSO₄·7H₂O, 1.5 % (w/v) Bacto agar (Oxoid), 50 % (v/v) natural seawater and 50 % (v/v) distilled water at pH 7.8. The isolation (by the replica-plating method) and purification procedures have been described elsewhere (Ivanova *et al.*, 1996). The strain was stored at –80 °C in marine broth 2216 (MB, Oxoid) supplemented with 20 % (v/v) glycerol.

The following physiological and biochemical properties were determined by methods described by Smibert & Krieg (1994): oxidation/fermentation of glucose, denitrification (Azegami *et al.*, 1987), oxidase and catalase activity, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, sodium requirement [0, 0.5, 1, 3, 5, 8, 10, 12 % (w/v) NaCl], indole and H₂S production and the ability to hydrolyse starch, Tween 80 and casein. The growth of the novel strain at different temperatures was determined in MB and on plates of Medium A after 24–72 h at 2, 4, 6, 29, 30, 35 and 37 °C. Biolog GN test kits were also used to examine the primary oxidation of 95 carbon compounds as described elsewhere (Ivanova *et al.*,

Abbreviation: FAMES, fatty acid methyl esters.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain Z 271^T is FJ535355.

Transmission electron micrographs of cells of strain Z 271^T and an extended phylogenetic tree are available as supplementary figures with the online version of this paper. A supplementary table detailing the cellular fatty acid composition of the novel strain is also available.

1998). Other biochemical tests were carried out with API 2NE and API ZYM strips (bioMérieux). To enable the isolation and characterization of bacteriochlorophyll *a*, strain Z 271^T was grown at 28 °C on marine agar 2216 (MA, Oxoid) over 48 h and the bacterial mass was collected from five Petri plates as described elsewhere (Ivanova *et al.*, 2004, 2005a). Cell morphology was examined by transmission electron microscopy (Libra 120; Carl Zeiss) using cells from 1, 4, and 8 day cultures on MA at 28 °C. A drop of particle-free (autoclaved and ultracentrifuged) distilled water was placed on the growth of a 24 h-culture. A 30 µl sample of the resulting bacterial suspension was applied to carbon- and Formvar-coated 400-mesh copper grids. A drop of 1.25 % (w/v) uranyl acetate was added and the bacteria were allowed to adhere for 1 min at room temperature. Superfluous liquid was gently removed by using a piece of filter paper.

Phenotypic analyses showed that strain Z 271^T was Gram-negative, strictly aerobic, oxidase- and catalase-positive, formed buds, produced polyhydroxybutyrate granules as storage material (See Supplementary Fig. S1 in IJSEM Online), produced H₂S and indole and did not show a tendency towards denitrification. The morphological and physiological properties of strain Z 271^T are given in Table 1 and in the species description.

To determine the phospholipid and fatty acid contents, cell biomass of strain Z 271^T and *G. antarcticus* IMCC3135^T was harvested from agar plates after cultivation for 48 h on MA at 28 °C. Lipids were extracted according to the method described by Bligh & Dyer (1959). Polar lipids were separated and identified as previously described (Ivanova *et al.*, 2005b). The lipids were treated with 5 % HCl in methanol at 80 °C for 180 min to produce fatty acid methyl esters (FAMES) (Christie, 1982). FAMES were analysed by FID-GC (GC-17; Shimadzu) with a fused silica

capillary column (30 m × 0.25 mm) coated with Supelcowax 10 at 210 °C. Helium was used as the carrier gas. FAMES were identified by comparing the retention times with those of authentic standards and using equivalent chain-length measurements. To ensure correct identification, FAMES were analysed by GC-MS (GCMS0QP5050A; Shimadzu) fitted with an MDN-5S capillary column (30 m × 0.25 mm). The column was programmed to hold the temperature at 170 °C for 1 min, increase it to 240 °C at 2 °C min⁻¹ and to then hold it at 240 °C for 20 min. The temperature of the injector and detector was 250 °C. Phosphatidylethanolamine (36.9 %) and phosphatidylglycerol (63.1 %) were found to be the major constituents of the phospholipids. Traces of diphosphatidylglycerol were also detected, however no glycopospholipids were detected. The major fatty acids formed by strain Z 271^T were C_{18:1}ω7c (43.7 %), C_{16:1}ω7c (31.1 %) and C_{16:0} (16.8 %) (see Supplementary Table S1 in IJSEM Online). The isoprenoid quinone composition was characterized by HPLC-MS (1200L; Varian) using a reverse phase type Varian Omnisphere 3 C18 column (20 cm × 2 mm) and acetonitrile as the mobile phase at a flow rate of 0.5 ml min⁻¹. The column was held at a temperature of 55 °C. Menaquinones were detected by UV-visible analysis at a wavelength of 270 nm.

The 16S rRNA gene sequence was amplified and sequenced by the Australian Genome Research Facility (AGRF) Laboratories (Brisbane, Australia). The 16S rRNA gene sequence (GenBank accession no. FJ535355) of strain Z 271^T was analysed by BLAST against a database of cultured species (<http://bioinfo.unice.fr/blast>) and the EzTaxon database of bacterial type strains (<http://147.47.212.35:8080>) in order to retrieve the 80 most similar sequences. These sequences were then aligned with CLUSTAL W2 (Larkin *et al.*, 2007). Alignments were then manually checked with

Table 1. Characteristics that differentiate strain Z 271^T and *G. antarcticus* IMCC3135^T

Strains: 1, Z 271^T; 2, *G. antarcticus* IMCC3135^T. Both species are catalase- and oxidase-positive, coccoid cells motile by means of tuft flagella. The NaCl range for growth is between 0.5 % and 5 %. Both strains accumulate polyhydroxybutyrate and do not produce bacteriochlorophyll *a* or pigments. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2
Bud formation	+	–
Temperature optimum (°C)	25	20
pH optimum	7.5	7
NaCl optimum (% w/v)	2.5	2.0
Hydrolysis of:		
Alkaline phosphatase	–	+
Gelatin	w	+
Lipase (C14)	+	–
Assimilation of:		
L-Fructose, L-proline, L-threonine, putrescine, 2-aminoethanol, DL-α-glycerol phosphate	–	+
α-Cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, L-arabinose, D-arabitol, cellobiose, D-mannitol, D-ribose, sucrose, D-xylose, acetic acid, inosine, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate	+	–
DNA G + C content (mol%)	60.2	57.5

SEAVIEW (Galtier *et al.*, 1996) and domains common to all sequences were used to derive an initial phylogenetic tree using the most recent version of SEAVIEW (see Supplementary Fig. S2 in IJSEM Online). This phylogenetic tree enabled the new strain to be identified as most closely related to the genus *Granulosicoccus*.

Alignments were checked again and a full phylogenetic analysis was undertaken using the neighbour-joining (BIONJ) and maximum-likelihood algorithms, excluding positions containing indels. For the neighbour-joining analysis, a distance matrix was calculated using the Kimura two-parameter correction. Bootstraps were performed using 1000 replications. BIONJ was used according to the method described by Gascuel (1997) and maximum-likelihood was determined using PhyML (Guindon & Gascuel, 2003). The phylogenetic trees were drawn using TreeDyn (Chevenet *et al.*, 2006) or SEAVIEW. The new strain formed a very robust cluster (confirmed by all methods) only with *G. antarcticus* IMCC3135^T (100% bootstrap), showing 18 differences over 1480 nt. The 16S rRNA gene sequence similarity between strain Z 271^T and *G. antarcticus* was 98.8% and the novel strain was thus identified as a member of the genus *Granulosicoccus* (Fig. 1).

DNA was isolated from the new strain according to the method described by Marmur (1961). The G + C content of the DNA was determined using the thermal denaturation method of Marmur & Doty (1962). The G + C content of strain Z 271^T was 60.2 ± 0.5 mol%. DNA–DNA hybridization was performed spectrophotometrically as described by De Ley *et al.* (1970). Strain *G. antarcticus* IMCC3135^T was kindly provided by Dr Jang-Cheon Cho

and Dr Lee Kiyong. DNA from strain Z 271^T showed 35% DNA–DNA hybridization with *G. antarcticus* IMCC3135^T. This value was far lower than 70%, which is the cut-off value recommended for the definition of a genomic species (Wayne *et al.*, 1987), and clearly indicated that strain Z 271^T represents a genomic species that is separate from *G. antarcticus* IMCC3135^T.

In summary, strain Z 271^T could be easily distinguished from *G. antarcticus* IMCC3135^T by a number of phenotypic traits. The two strains showed distinct phenotypic profiles as regards their metabolic activity as determined with the API ZYM system and in the assimilation of carbon substrates with Biolog GN microplates (Table 1). In addition, strain Z 271^T could be distinguished by its particular cell morphology (bud formation), its optimal temperature for growth and the different proportions of major fatty acids, e.g. C_{18:1}ω7c and C_{16:0}. Thus, on the basis of characteristic phenotypic, chemotaxonomic and well-confirmed phylogenetic evidence, strain Z 271^T represents a novel species of the genus *Granulosicoccus* for which the name *Granulosicoccus coccoides* sp. nov. is proposed.

Description of *Granulosicoccus coccoides* sp. nov.

Granulosicoccus coccoides [coc.co'i.des, N.L. n. *coccus* (from Gr. n. *kokkos*) grain, seed; L. suff. -*oides* (from Gr. suff. *eides*, from Gr. n. *eidos* that which is seen, form, shape, figure) resembling, similar; N.L. masc. adj. *coccoides* similar to a round, ball-shaped cell morphology].

Round-shaped cells, single and about 1 µm in diameter. Gram-negative. Motile by means of tuft flagella, forms

Fig. 1. Phylogenetic position of strain Z 271^T according to 16S rRNA gene sequence analysis. The tree is an extract of a larger phylogenetic tree obtained after a distance analysis (See Supplementary Fig. S2). Bootstrap percentages (1000 replications) are indicated for branches when greater than 50%. Asterisks indicate that branches were also retrieved using a maximum-likelihood analysis. Bar, 0.02 substitutions per site.

buds. Aerobic. Chemorganotrophic with respiratory metabolism. Colonies are uniformly round, 0.5–1 mm in diameter, regular and convex on MA or Medium A. Does not form endospores. Forms polyhydroxybutyrate granules. Oxidase- and catalase-positive. Requires Na^+ ions or seawater for growth. Growth occurs in media with 0.5–5 % (w/v) NaCl. Temperature for growth ranges from 5 to 28 °C. No growth is detected at 35 °C. The pH range for growth is 6.0–10.0, with an optimum at 7.5–8.0. Negative for indole, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and H_2S production. Decomposes Tweens 20, 40 and 80, weakly decomposes gelatin. Utilizes the following carbon sources (Biolog): α -cyclodextrin, β -cyclodextrin, dextrin, glycogen, inulin, mannan, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α -D-glucose, *myo*-inositol, α -D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-ribose, sucrose, D-xylose, acetic acid, β -hydroxybutyric acid, α -ketovaleic acid, lactamide, D-lactic acid methyl ester, L-lactic acid, mono-methyl succinate, propionic acid, pyruvic acid, succinamic acid, succinic acid, L-serine, inosine, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate. According to API ZYM strips, produces esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl- β -glucosaminidase. According to API 20NE strips, produces β -glucosidase and *para*-nitrophenyl- β -D-galactopyranosidase. The predominant phospholipids are phosphatidylethanolamine (36.9 %) and phosphatidylglycerol (63.1 %). The main cellular fatty acids are $\text{C}_{18:1\omega7c}$ (43.7 %), $\text{C}_{16:1\omega7c}$ (31.1 %) and $\text{C}_{16:0}$ (16.8 %). The major isoprenoid quinone is Q-8.

The type strain, Z 271^T (=KMM 6014^T=CIP 109923^T), was isolated from the surface of leaves of *Zostera marina* grown in Troitz Bay, Gulf of Peter the Great, Pacific Ocean. The DNA G + C content of the type strain is 60.2 mol%.

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