Miniimonas arenae gen. nov., sp. nov., an actinobacterium isolated from sea sand

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A Gram-positive, non-motile, coccoid- to rod-shaped, non-spore-forming bacterium, designated strain YM18-15^T, was isolated from sea sand and studied using a polyphasic taxonomic approach. Strain YM18-15^T grew under both aerobic and anaerobic conditions. The cell-wall peptidoglycan type was A4 β and ornithine was the diagnostic diamino acid. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and an unknown phospholipid, MK-8(H₄) was the major menaquinone and the predominant fatty acids were anteiso-C_{15:0} and C_{16:0}. The DNA G + C content was 74.2 mol%. High 16S rRNA gene sequence similarities (96.3–97.3 %) were found with the sequences of the type strains of the three genera of the family *Beutenbergiaceae*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YM18-15^T formed a clade with *Serinibacter salmoneus*, *Salana multivorans* and *Beutenbergia cavernae*. Strain YM18-15^T differed from these three type strains in chemotaxonomic characteristics and in 16S rRNA gene signature nucleotides. Based on genetic and chemotaxonomic evidence, it is suggested that strain YM18-15^T represents a novel species of a new genus within the family *Beutenbergiaceae*, for which the name *Miniimonas arenae* gen. nov., sp. nov. is proposed. The type strain of the type species is YM18-15^T (=NBRC 106267^T=KCTC 19750^T=MBIC 08348^T).

The family *Beutenbergiaceae* within the suborder *Micrococcineae* was proposed by Zhi *et al.* (2009). The family *Beutenbergiaceae* contains three genera, *Salana* (von Wintzingerode *et al.*, 2001), *Beutenbergia* (Groth *et al.*, 1999) and *Serinibacter* (Hamada *et al.*, 2009). Each of these genera is represented by only one species. *Salana multivorans* DSM 13521^T was isolated from an anaerobic bioreactor, *Beutenbergia cavernae* DSM 12333^T was isolated from a cave and *Serinibacter salmoneus* DSM 21801^T was isolated from the intestinal tract of a fish. In the present study, we describe a bacterium, strain YM18-15^T, which was isolated from sea sand in Teguma fishing harbour in Nagasaki Prefecture, Japan. It is proposed that strain YM18-15^T represents a novel species in a new genus within the family *Beutenbergiaceae*.

Isolation was performed on H medium (for composition, see Supplementary Method in IJSEM Online) at 25 °C for 30 days. Strain YM18-15^T was maintained as glycerol

suspensions (20 %, v/v) at –80 °C. Biomass for phenotypic tests was obtained from Luria–Bertani (LB) broth (Difco) by incubating at 30 °C for 1 week. Biomass for chemical and molecular systematic studies was obtained by culturing the strain in IL8 medium (10 g lactose, 10 g polypeptone, 15 g yeast extract, 10 g NaCl), except for the cells used for the preparation of fatty acids. For quantitative analysis of whole-cell fatty acids, cells were grown on tryptic soy agar (TSA) at 30 °C for 7 days. Gram staining and tests for motility were performed with the Favor G (Nissui) and API M media (bioMérieux), respectively, according to the manufacturers' instructions.

Morphology was observed using light microscopy (ECLIPSE E600; Nikon). Colony morphology and colour were observed on LB agar (Difco) at 30 °C for 1 week. Growth was tested at 5, 10, 15, 20, 25, 28, 35, 37, 42, 45 and 50 °C on LB agar. Experiments investigating tolerance to NaCl were carried out using LB broth as the basal medium. The following NaCl concentrations (w/v) were tested: 0.5, 2, 5, 8, 10, 15 and 20 %. For determination of the optimal pH for growth, cells were grown on LB agar at pH 5–11 at 30 °C for 3 weeks. The ability of the novel strain to use sole

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Supplementary material is available with the online version of this paper.

carbon sources was tested with International *Streptomyces* Project (ISP) medium 9 (Difco) supplemented with 1% (w/v) carbon source for 3 weeks. The activity of several enzymes was determined by using API ZYM (bioMérieux) and checked after 24 h. Several physiological tests were performed using the API 20NE kit (bioMérieux). Acid production under anaerobic conditions was assayed using API 20A (bioMérieux). API tests were performed twice. The oxidase reaction was tested by using cytochrome oxidase test paper (Nissui Pharmaceutical Co.). Catalase activity was determined based on production of bubbles after the addition of a drop of 3% H₂O₂. All phenotypic assays (except analysis of the temperature range) were carried out at 30 °C, which was in the range of optimal growth temperatures for the strain.

Menaquinones were extracted using the procedures described by Collins *et al.* (1977) and analysed by LCMS as described by Ogiso *et al.* (2008), but without adding phosphoric acid to the elution buffer. Polar lipids were extracted according to Minnikin *et al.* (1984) and analysed by TLC. The purified cell wall was obtained by using the method described by Schleifer & Kandler (1972). Amino acids in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates (Merck) by using the solvent systems of Harper & Davis (1979) and by HPLC as their phenylthiocarbamoyl derivatives with a Wakopak WS-PTC column (Wako Pure Chemical Industries) (Yokota *et al.*, 1993). Analysis of the whole-cell fatty acid pattern was performed by using the MIDI system (Microbial ID, Inc.). Mycolic acids were analysed by the method of Minnikin *et al.* (1975). The acyl type of the muramic acid in the cell wall was determined according to the method described by Uchida *et al.* (1999). Analysis of sugars from whole-cell hydrolysates was performed using the procedures described by Stanek & Roberts (1974). DNA was prepared according to the method of Suzuki *et al.* (1999). The DNA base composition of strain YM18-15^T was determined by reversed-phase HPLC using a DNA-GC kit (Yamasa) according to the method of Kumagai *et al.* (1988).

An approximately 1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers 27F and 1492R (*Escherichia coli* numbering system; Weisburg *et al.*, 1991) specific to the 16S rRNA gene. A sequence similarity search was conducted using GenBank BLASTN (National Center for Biotechnology Information). Sequences of closely related taxa were retrieved and aligned using the CLUSTAL_X program (Thompson *et al.*, 1997) and the alignment was corrected manually. Aligned sequences were analysed by using MEGA 4.0.2 software (Tamura *et al.*, 2007). For neighbour-joining analysis (Saitou & Nei, 1987), genetic distances were calculated using Kimura's two-parameter model (Kimura, 1983). Clustering with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods was determined by using bootstrap values based on

1000 replications (Felsenstein, 1985). Similarity values were calculated using the MEGA software.

The components of the peptidoglycan of strain YM18-15^T were Ala, Ser, Glu and Orn in a molar ratio of 0.4:0.5:1.0:0.4 and the peptidoglycan type was A4β (Schleifer & Kandler, 1972) with an interpeptide bridge of Orn–Glu. The fatty acid pattern was of the iso- and anteiso-branched and straight-chain saturated type. Major fatty acids were anteiso-C_{15:0} (46.7%) and C_{16:0} (23.2%) and minor fatty acids were anteiso-C_{17:0} (9.4%), C_{14:0} (5.4%), iso-C_{16:0} (3.8%), iso-C_{15:0} (3.4%), iso-C_{14:0} (2.8%), C_{15:0} (2.3%) and iso-C_{17:0} (1.5%). The detailed fatty acid composition of strain YM18-15^T is given in Supplementary Table S1 (see IJSEM Online). The isoprenoid quinones were menaquinones of type MK-8(H₄) (88.5%), MK-8 (7.0%), MK-8(H₂) (2.7%) and MK-8(H₆) (1.8%). The polar lipids consisted of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The DNA G + C content was 74.2 mol%.

Phylogenetic analysis based on the neighbour-joining method showed that strain YM18-15^T was positioned within the family *Beutenbergiaceae* of the suborder *Micrococcineae* and joined the phylogenetic lineage of *Serinibacter salmoneus* DSM 21801^T (Fig. 1). The relative position of strain YM18-15^T was also confirmed in the maximum-parsimony tree. The BLAST sequence similarity result based on the 16S rRNA gene sequence revealed that strain YM18-15^T was closely related to *Serinibacter salmoneus* DSM 21801^T (sequence similarity 97.3%), *Salana multivorans* DSM 13521^T (96.3%) and *B. cavernae* DSM 12333^T (97.1%) of the family *Beutenbergiaceae*.

The differences in cell morphology and chemotaxonomic characteristics between strain YM18-15^T and members of the genera *Serinibacter*, *Salana* and *Beutenbergia* are shown in Table 1. The most noticeable differences were in the polar lipids. The peptidoglycan type of the cell wall was A4β (containing Orn) for strain YM18-15^T, but A4α (containing Lys) for the genera *Serinibacter* and *Beutenbergia*. The combination of major fatty acids (anteiso-C_{15:0} and C_{16:0}) for strain YM18-15^T did not match those of the genera *Salana* or *Beutenbergia*. The results of detailed test reactions are presented in the species description and Supplementary Table S2 (available in IJSEM Online). The pattern of the 16S rRNA gene sequence signature nucleotides is shown in Supplementary Table S3. The pattern of 16S rRNA signature nucleotides consisted of nucleotides at positions 140:223 (G–C), 589:650 (U–A), 610 (U), 612:628 (C–G), 616:624 (G–C), 839:847 (C–A) and 863 (A), which indicated that strain YM18-15^T did not belong to any recognized genus within the family *Beutenbergiaceae*, having signature nucleotide differences from *Serinibacter salmoneus* DSM 21801^T (three positions), *Salana multivorans* DSM 13521^T (three positions) and *B. cavernae* DSM 12333^T (four positions). The pattern of 16S rRNA gene signature nucleotides of strain YM18-15^T perfectly matched that given in the description of the family *Beutenbergiaceae* as reported by Hamada *et al.*

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences (1368 bases), for strain YM18-15^T, all the members of the family *Beutenbergiaceae* and type strains from related genera within the suborder *Micrococcineae*. Only values with >50% significance are shown. Asterisks indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. *Kineosporia aurantiaca* NRRL B-16913^T was used as an outgroup. Bar, 0.01 K_{nuc} .

(2009). Strain YM18-15^T and the genera *Beutenbergia*, *Salana* and *Serinibacter* had an A–G at position 131:231, not a C–G as reported by Zhi *et al.* (2009). From the differences in its chemotaxonomic and genotypic properties, strain YM18-15^T is considered to represent a novel genus of the family *Beutenbergiaceae*, for which the name *Miniimonas arenae* gen. nov., sp. nov. is proposed.

Description of *Miniimonas* gen. nov.

Miniimonas (Mi.ni.i.mo'nas. L. adj. *minius* cinnabar-red, vermilion; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Miniimonas* vermilion monad, referring to the colour of the cell mass).

Gram-positive, non-motile, coccoid- to rod-shaped, oxidase-negative and catalase-positive. Growth occurs under both aerobic and anaerobic conditions (i.e. facultatively anaerobic). The major menaquinone is MK-8(H₄). The fatty acid pattern is of the iso- and anteiso-branched and straight-chain

saturated type. The major fatty acids are anteiso-C_{15:0} and C_{16:0}. The cell-wall peptidoglycan contains Orn, Ser, Ala and Glu. The whole-cell sugars are galactose, xylose and ribose. Mycolic acids are absent. The acyl type of muramic acid is acetyl. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and one unknown phospholipid. Phylogenetically, the genus *Miniimonas* belongs to the family *Beutenbergiaceae* of the suborder *Micrococcineae* of the class *Actinobacteria*. The type species is *Miniimonas arenae*.

Description of *Miniimonas arenae* sp. nov.

Miniimonas arenae (a.re'na.e. L. gen. n. *arenae* of sand, isolated from sea sand).

Displays the following properties in addition to those given in the genus description. Rod-shaped cells are 0.6–3.7 µm in length. Cells exhibit a rod-coccus cycle. The diameters of

Table 1. Cell morphology and differential chemotaxonomic characteristics of strain YM18-15^T and type strains of the genera *Serinibacter*, *Salana* and *Beutenbergia*

Taxa: 1, YM18-15^T; 2, *Serinibacter salmoneus* DSM 21801^T (data from Hamada *et al.*, 2009); 3, *Salana multivorans* DSM 13521^T (von Wintzingerode *et al.*, 2001); 4, *Beutenbergia cavernae* DSM 12333^T (Groth *et al.*, 1999). The major menaquinone was MK-8(H₄) for all taxa. PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PL(s), unknown phospholipid(s).

Characteristic	1	2	3	4
Cell morphology	Rod, coccoid	Short rods, irregular	Rod, coccoid, club-like	Rod/coccus cycle, non-sporulating
Major fatty acids	anteiso-C _{15:0} , C _{16:0}	anteiso-C _{15:0} , C _{16:0}	anteiso-C _{15:0} , C _{14:0}	iso-C _{15:0} , anteiso-C _{15:0}
Polar lipids	PG, DPG, PI, 1 PL	PE, PG	PG, DPG, PLs	PI, DPG, 3 PLs
Cell-wall amino acid				
Diamino acid	Orn	Lys	Orn	Lys
Peptidoglycan type	A4β	A4α	A4β	A4α
DNA G + C content (mol%)	74	71	75	71

the cocci are 1.0–1.7 µm. Growth occurs weakly at 25–30 °C. The pH range for growth is 5–11, with optimum growth at pH 7–7.5. Cells grow in the absence of NaCl, but tolerate up to 5 % NaCl (w/v). When grown aerobically for 7 days on LB agar or IL8 medium agar, the type strain forms a vermilion-coloured cell mass. Does not form spores. Cells form circular and smooth colonies that are 1–4 mm diameter after 7 days at 30 °C on LB agar. Nitrate is reduced to nitrite. H₂S is not produced. Hydrolysis of starch is negative. The type strain forms vermilion-coloured colonies. Based on API ZYM tests, cells are positive for esterase lipase (C8), leucine arylamidase, naphthol AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase, but negative for alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Cells are weakly positive for esterase (C4) and acid phosphatase. Utilizes the following substrates as sole carbon sources: L-arabinose, D-mannose, D-xylose, D-glucose, D-fructose, L-rhamnose and maltose monohydrate, but does not utilize D-mannitol. Anaerobic formation of acid from API 20A is positive for glucose, sucrose, maltose, D-xylose, L-arabinose, D-mannose, raffinose, L-rhamnose and trehalose, weakly positive for cellobiose and negative for D-mannitol, lactose, salicin, glycerol, melezitose and D-sorbitol. Based on API 20A tests, cells are anaerobically positive for the hydrolysis of aesculin, but negative for the hydrolysis of gelatin and for the production of indole, arginine dihydrolase and urease. The peptidoglycan type, polar lipids, fatty acid pattern and major menaquinone are as given in the genus description.

The type strain, YM18-15^T (=NBRC 106267^T=KCTC 19750^T=MBIC 08348^T), was isolated from sea sand in Teguma fishing harbour in Nagasaki Prefecture, Japan. The G+C content of the genomic DNA is 74.2 mol%.

Emended description of the family *Beutenbergiaceae* Zhi *et al.* 2009 emend. Hamada *et al.* 2009

The description of the family is as emended by Hamada *et al.* (2009), but the 16S rRNA gene sequence signature nucleotide pattern contains an A–G at position 131:231, not a C–G (Zhi *et al.*, 2009). The family contains the type genus *Beutenbergia* (Groth *et al.*, 1999), as well as the genera *Salana* (von Wintzingerode *et al.*, 2001), *Serinibacter* (Hamada *et al.*, 2009) and *Miniimonas*.

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