Demequina flava sp. nov. and *Demequina sediminicola* sp. nov., isolated from sea sediment

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Two novel Gram-stain-positive bacteria, designated HR08-7^T and HR08-43^T, were isolated from a sea sediment sample from Rishiri Island, Hokkaido, Japan, and their taxonomic positions were investigated by a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strains HR08-7^T and HR08-43^T and the members of the genus *Demequina* formed a monophyletic cluster with similarity range of 95.5–99.0 %. The peptidoglycan type of strains HR08-7^T and HR08-43^T was A4 β . The predominant menaquinone of both strains was demethylmenaquinone DMK-9(H₄) and the major fatty acid was anteiso-C_{15:0}. The DNA G + C contents of strains HR08-7^T and HR08-43^T were 64.5 and 62.4 mol%, respectively. The results of phylogenetic analysis and DNA–DNA hybridization, along with differences of strains HR08-7^T and HR08-43^T from the recognized *Demequina* species in phenotypic characteristics, indicate that the two strains merit classification as representatives of two novel species of the genus *Demequina*, for which the names *Demequina flava* sp. nov. and *Demequina sediminicola* sp. nov. are proposed; the type strains are HR08-7^T (=NBRC 105854^T=DSM 24865^T) and HR08-43^T (=NBRC 105855^T=DSM 24867^T), respectively.

The family *Demequinaceae* proposed by Ue *et al.* (2011) currently contains two genera; *Demequina* and *Lysinimbium* (Hamada *et al.*, 2012). The genus *Demequina* was first proposed by Yi *et al.* (2007) and its description was later emended by Ue *et al.* (2011). At the time of writing, the genus *Demequina* consisted of six species: *Demequina aestuarii* (Yi *et al.*, 2007; type species of the genus), *D. lutea* (Finster *et al.*, 2009), *D. salsinemoris* (Matsumoto *et al.*, 2010), *D. globuliformis*, *D. oxidasica* and *D. aurantiaca* (Ue *et al.*, 2011). The genus *Demequina* is characterized by the presence of a discriminative menaquinone, namely, demethylmenaquinone DMK-9(H₄). In addition, the members of this genus have peptidoglycan type A4 β with ornithine as the diagnostic diamino acid.

During the course of a study of bacterial diversity in cool-temperate and subtropical areas, two novel actinobacteria were isolated from a sea sediment sample collected from a cool-temperate island in Japan. Comparative 16S rRNA gene sequence analysis revealed that the isolates are

phylogenetically related to members of the genus *Demequina*. The objective of this study was to determine the taxonomic positions of the isolates by using a polyphasic approach.

A sea sediment sample was collected in Rishiri Island, Hokkaido, Japan. Approximately 1 g of the sample was diluted 10-, 100- and 1000-fold with saline before 0.2 ml of each dilution was spread on plates of 1/5 NBRC medium 802 (0.2 % polypeptone, 0.04 % yeast extract, 0.02 % MgSO₄·7H₂O and 1.5 % agar; pH 7.0) supplemented with 5.0 % NaCl, 0.005 % cycloheximide and 0.002 % nalidixic acid. After cultivation at 30 °C for 1 week and repeated isolation, strains HR08-7^T and HR08-43^T were obtained. As both strains did not require NaCl for growth, NBRC medium 802 (1.0 % polypeptone, 0.2 % yeast extract, 0.1 % MgSO₄·7H₂O and 1.5 % agar if required; pH 7.0) was used as basal medium for this study. Biomass for chemotaxonomic and molecular systematic studies, except for cellular fatty acids analysis, was obtained by incubating the strains in shake flasks for 48 h at 28 °C at 100 r.p.m. *D. globuliformis* NBRC 106266^T and *D. salsinemoris* NBRC 105323^T were used as reference strains in this study.

Colony appearance was examined after incubation at 28 °C for 3 days. Morphological features were observed

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HR08-7^T and HR08-43^T are AB674956 and AB674957, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

for up to 7 days of incubation under a light microscope (BX-51; Olympus) and a scanning electron microscope (JSM-6060; JEOL). Cell motility, growth parameters (temperature, pH range and NaCl tolerance), anaerobic growth, Gram staining and oxidase activity were determined using the methods described by Hamada *et al.* (2010). Other physiological and biochemical tests were performed using API ZYM, API Coryne and API 50CH systems (bioMérieux) according to the manufacturer's instructions.

Strains HR08-7^T and HR08-43^T formed yellow, circular, transparent, smooth colonies that were approximately 0.5–1.0 mm in diameter after 3 days cultivation. The cells were irregular, rod-shaped (0.5–0.8 × 1.0–5.0 µm), Gram-stain-positive, non-motile and non-spore-forming. Both strains grew at 10–28 °C. No growth was observed at 5, 37, 45 or 60 °C. The pH ranges for growth of strains HR08-7^T and HR08-43^T were 7.0–9.0 and 7.0–10.0, respectively. Optimal growth of both strains was noted at 28 °C and pH 7.0–8.0. Both strains exhibited good growth with NaCl concentrations of 0–7 % and moderate growth with 10 %; no growth was observed with 15 % (w/v) NaCl. Optimal growth was noted with 0 % NaCl. Moderate growth was observed under anaerobic conditions. The results of other physiological and biochemical analyses are summarized in the species descriptions below.

PCR amplification and 16S rRNA gene sequencing of strains HR08-7^T and HR08-43^T were performed as described previously (Hamada *et al.*, 2010). The phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated using EzTaxon-e (Kim *et al.*, 2012). The almost-complete 16S rRNA gene sequences of the strains were aligned with reference sequences of members of the genus *Demequina* and some related taxa by using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms using the MEGA 5.01 program (Tamura *et al.*, 2011). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

Phylogenetic analysis, based on 16S rRNA gene sequences, revealed that strains HR08-7^T and HR08-43^T formed a monophyletic cluster with the members of the genus *Demequina* (Fig. 1). Strains HR08-7^T and HR08-43^T exhibited 16S rRNA gene sequence similarities as follows: 99.0 and 98.4 % with *D. globuliformis* NBRC 106266^T and 97.6 and 97.5 % with *D. salsinemoris* NBRC 105323^T, respectively. Both strains showed less than 97.2 % similarity with other members of the genus *Demequina*. The similarity value between strains HR08-7^T and HR08-43^T was 98.7 %.

Amino acids and the isomers in cell-wall hydrolysates were analysed as described previously (Hamada *et al.*, 2012). Analysis of cell-wall sugars was carried out using the

method described by Mikami & Ishida (1983) using HPLC (LC-10AD; Shimadzu). The preparation and analysis of cellular fatty acid methyl esters were performed using the protocol of the MIDI Sherlock Microbial Identification System (Sasser, 1990) and GC (model 6890N; Agilent Technologies) with Sherlock MIDI software (version 4.0) and a TSBA database (version 4.0). Biomass grown on tryptic soy agar (Difco) for 24 h at 28 °C was used for cellular fatty acids analysis. Isoprenoid quinones, polar lipids and DNA G+C content were analysed as described by Hamada *et al.* (2010).

The obtained peptidoglycan samples of strains HR08-7^T and HR08-43^T contained alanine (Ala), glutamic acid (Glu), serine (Ser) and ornithine (Orn) at molar ratios of 0.9:2.0:1.7:0.9 and 0.8:2.0:1.6:0.8, respectively. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of D-Ala, D-Glu, L-Ser and L-Orn. Additionally, strain HR08-43^T contained a significant amount of D-Ser. Meanwhile, L-Ala was not detected in either strain. These data suggested that the cell-wall peptidoglycans of strains HR08-7^T and HR08-43^T are of the A4β type (Schleifer & Kandler, 1972) with an interpeptide bridge comprising L-Ser and D-Glu (HR08-7^T) or D-Ser and D-Glu (HR08-43^T) and an L-Ser residue at position 1 of the peptide subunit of both strains. The cell-wall sugars of strain HR08-7^T were galactose, mannose and ribose, while those of strain HR08-43^T were mannose, ribose and xylose. The only isoprenoid quinone detected was demethylmenaquinone DMK-9(H₄). The major cellular fatty acids of strain HR08-7^T were anteiso-C_{15:0} (60.7 %) and anteiso-C_{17:0} (11.0 %), while minor components were C_{18:0} (6.6 %), C_{16:0} (5.5 %), C_{14:0} (3.7 %), iso-C_{16:0} (3.4 %), anteiso-C_{15:1} A (3.2 %), C_{17:0} (1.8 %), C_{15:0} (1.7 %), iso-C_{14:0} (1.5 %) and iso-C_{15:0} (1.1 %). The major cellular fatty acid of strain HR08-43^T was anteiso-C_{15:0} (65.9 %), while minor components were anteiso-C_{17:0} (8.6 %), C_{16:0} (4.2 %), C_{14:0} (4.0 %), iso-C_{16:0} (3.0 %), C_{15:0} (3.0 %), C_{18:0} (2.5 %), iso-C_{14:0} (2.4 %), iso-C_{15:0} (1.8 %), anteiso-C_{15:1} A (1.8 %), C_{17:0} (1.5 %) and C_{13:0} (1.2 %). The principal polar lipids of strains HR08-7^T and HR08-43^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannoside and an unidentified phosphorus-free polar lipid, along with minor or trace amounts of two additional unidentified phosphorus-free polar lipids (see Fig. S1 available in IJSEM Online). In addition, strain HR08-43^T possessed one glycolipid as a major component, while strain HR08-7^T possessed one glycolipid as a minor component. The DNA G+C contents of strains HR08-7^T and HR08-43^T were 64.5 and 62.4 mol%, respectively.

The microplate hybridization method developed by Ezaki *et al.* (1988, 1989) was used to determine DNA relatedness. Hybridizations were performed using five replications. After the highest and lowest values for each sample were excluded, the mean was reported as the DNA–DNA relatedness value. Strains HR08-7^T and HR08-43^T showed

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences of strains HR08-7^T and HR08-43^T and their taxonomic neighbours, constructed with the neighbour-joining method. The sequence of *Brevibacterium linens* DSM 20425^T (GenBank accession number X77451) was used as the outgroup. Bootstrap values (>70%) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 K_{nuc} .

low DNA–DNA relatedness values with *D. globuliformis* NBRC 106266^T (2–5% and 2–4%) and *D. salsinemoris* NBRC 105323^T (5–6% and 1–2%), and the DNA–DNA relatedness value between strains HR08-7^T and HR08-43^T was 2–7% (see Table S1). These values are well below the 70% cut-off point of DNA relatedness, which is a criterion for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987). Moreover, the DNA–DNA hybridization test was repeated twice with similar results.

The result of the phylogenetic analysis of the 16S rRNA gene sequence suggested that strains HR08-7^T and HR08-43^T belonged to the genus *Demequina*. The predominant menaquinone of the strains was demethylmenaquinone DMK-9(H₄); the peptidoglycan type was A4 β with ornithine as the diagnostic cell-wall diamino acid. These chemotaxonomic characteristics corresponded to those of the genus *Demequina*. Meanwhile, the DNA–DNA relatedness among strains HR08-7^T and HR08-43^T and related *Demequina* species were low, and the results of the physiological and biochemical tests distinguished strains HR08-7^T and HR08-43^T from related *Demequina* species (Table 1). Therefore, it is proposed that strains HR08-7^T and HR08-43^T be classified as representatives of two novel species of the genus *Demequina*, with the names *Demequina flava* sp. nov. and *Demequina sediminicola* sp. nov., respectively.

Description of *Demequina flava* sp. nov.

Demequina flava (fla'va. L. fem. adj. *flava* golden yellow, referring to the colour of the colonies).

Cells are irregular, rod-shaped, 0.5–0.8 $\times$ 1.0–5.0 μ m, Gram-stain-positive, facultatively anaerobic, non-motile and non-spore-forming. Colonies are smooth, yellow, circular and transparent. Catalase-positive, oxidase-negative. Grows at 10–28 $^{\circ}$ C (optimal 28 $^{\circ}$ C) and pH 7.0–9.0 (optimal pH 7.0–8.0). Growth occurs with NaCl concentrations of 0–10% (w/v) but not with 15%; optimal NaCl concentration is 0%. Acid phosphatase, esterase (C4), esterase lipase (C8), α -glucosidase, β -glucosidase, leucine arylamidase and phosphohydrolase are present, whereas *N*-acetyl- β -glucosaminidase, alkaline phosphatase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, lipase (C14), α -mannosidase, pyrazinamidase, pyrrolidonyl arylamidase, trypsin, urease and valine arylamidase are not. Aesculin is hydrolysed while gelatin is not. Nitrate is not reduced. Acid is produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, maltose, D-mannose, methyl β -D-xylopyranoside, salicin and D-xylose. Conversely, acid is not produced from *N*-acetylglucosamine, D-adonitol, amygdalin, D-arabinose, D- or L-arabitol, arbutin, dulcitol, erythritol, D- or L-fucose, gluconate, glycerol, glycogen, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, D-lactose, D-lyxose, D-mannitol, melezitose,

Table 1. Differential phenotypic characteristics of strains HR08-7^T and HR08-43^T and *Demequina* species

Strains: 1, strain HR08-7^T; 2, strain HR08-43^T; 3, *D. globuliformis* NBRC 106266^T; 4, *D. salsinemoris* NBRC 105323^T. Data are from this study unless indicated. Data in parentheses were reported in the original descriptions of the type species by Ue *et al.* (2011) (*D. globuliformis*) and Matsumoto *et al.* (2010) (*D. salsinemoris*). +, Positive; –, negative; w, weakly positive; ND, no data available. Gal, galactose; Man, mannose; Rib, ribose; Xyl, xylose.

Characteristic	1	2	3	4
Colony colour	Yellow	Yellow	Cream-yellow	Pale yellow
Growth temperature range (°C)	10–28	10–28	4–37*	14–34†
pH range for growth	7–9	7–10	5–11*	6–9†
NaCl tolerance (%)	10	10	15*	8†
Nitrate reduction	–	–	+	–
Acid phosphatase	+	+	+	– (+)
<i>N</i> -Acetyl- β -glucosaminidase	–	+	+	–
Chymotrypsin	–	+	+	–
Esterase lipase (C8)	+	+	w (+)	– (+)
α -Galactosidase	–	–	+	– (+)
β -Galactosidase	–	–	+	+
α -Glucosidase	+	+	+	– (+)
Leucine arylamidase	+	+	+	– (+)
Pyrrolidonyl arylamidase	–	–	+	–
Acid production from:				
<i>N</i> -Acetylglucosamine	–	+	+	–
Amygdalin	–	+	+	– (+)
L-Arabinose	+	–	+	+
Arbutin	–	+	– (+)	– (+)
D-Lactose	–	+	+	–
D-Lyxose	–	+	–	–
D-Mannitol	–	+	–	–
Methyl β -D-xylopyranoside	+	+	–	+
L-Rhamnose	–	+	– (+)	+
Sucrose	–	+	+	–
Trehalose	–	+	+	–
Turanose	–	+	–	+
Interpeptide bridge of peptidoglycan	L-Ser, D-Glu	D-Ser, D-Glu	ND	ND
Cell-wall sugars	Gal, Man, Rib	Man, Rib, Xyl	ND	ND
DNA G + C content (mol%)	64.5	62.4	67.3*	70–72†

*Data from Ue *et al.* (2011).

†Data from Matsumoto *et al.* (2010).

melibiose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, raffinose, L-rhamnose, D-ribose, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol or L-xylose. The peptidoglycan is of the A4 β type with an interpeptide bridge comprising L-Ser and D-Glu and an L-Ser residue at position 1 of the peptide subunit. The cell-wall sugars are galactose, mannose and ribose. The only menaquinone is demethylmenaquinone DMK-9(H₄). The major cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The principal polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannoside and an unidentified phosphorus-free polar lipid.

The type strain, HR08-7^T (=NBRC 105854^T=DSM 24865^T), was isolated from a sea sediment sample from

Rishiri Island, Hokkaido, Japan. The DNA G + C content of type strain is 64.5 mol%.

Description of *Demequina sediminicola* sp. nov.

Demequina sediminicola (se.di.mi.ni'co.la. L. n. *sedimen* - inis sediment; L. suff. -cola inhabitant, dweller; N.L. n. *sediminicola* sediment-dweller, referring to the source of the type strain).

Cells are irregular, rod-shaped, 0.5–0.8 $\times$ 1.0–5.0 μ m, Gram-stain-positive, facultatively anaerobic, non-motile and non-spore-forming. Colonies are smooth, yellow, circular and transparent. Catalase-positive, oxidase-negative. Grows at 10–28 °C (optimal 28 °C) and pH 7.0–10.0 (optimal pH 7.0–8.0). Growth occurs with NaCl concentrations of

0–10 % (w/v) but not with 15 %; optimal NaCl concentration is 0 %. Acid phosphatase, *N*-acetyl- β -glucosaminidase, α -chymotrypsin, esterase (C4), esterase lipase (C8), α -glucosidase, β -glucosidase, leucine arylamidase and phosphohydrolase are present, whereas alkaline phosphatase, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, lipase (C14), α -mannosidase, pyrazinamidase, pyrrolidonyl arylamidase, trypsin, urease and valine arylamidase are not. Aesculin is hydrolysed while gelatin is not. Nitrate is not reduced. Acid is produced from *N*-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, D-lactose, D-lyxose, maltose, D-mannitol, D-mannose, methyl β -D-xylopyranoside, L-rhamnose, salicin, sucrose, trehalose, turanose and D-xylose. Conversely, acid is not produced from D-adonitol, D- or L-arabinose, D- or L-arabitol, dulcitol, erythritol, D- or L-fucose, gluconate, glycerol, glycogen, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, melezitose, melibiose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, raffinose, D-ribose, D-sorbitol, L-sorbose, starch, D-tagatose, xylitol or L-xylose. The peptidoglycan is of the A4 β type with an interpeptide bridge comprising D-Ser and D-Glu and an L-Ser residue at position 1 of the peptide subunit. The cell-wall sugars are mannose, ribose and xylose. The only menaquinone is demethylmenaquinone DMK-9(H₄). The major cellular fatty acid is anteiso-C_{15:0}. The principal polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannoside, one unidentified phosphorus-free polar lipid and one glycolipid.

The type strain, HR08-43^T (=NBRC 105855^T=DSM 24867^T), was isolated from a sea sediment sample from Rishiri Island, Hokkaido, Japan. The DNA G+C content of type strain is 62.4 mol%.

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