Demequina salsinemoris sp. nov., isolated on agar media supplemented with ascorbic acid or rutin

Atsuko Matsumoto, Kengo Nakai, Kurimi Morisaki, Satoshi Ōmura and Yōko Takahashi

Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Correspondence
Yōko Takahashi
ytakaha@lisci.kitasato-u.ac.jp

Three strains, KV-810^T, KV-811 and KV-816, were isolated from mangrove soil from a southern island in Japan on media supplemented with ascorbic acid or rutin. These strains contained L-ornithine as the diagnostic diamino acid in the cell-wall peptidoglycan and DMK-9(H_4) as the predominant menaquinone. The G+C content of the DNA was 70–72 mol%. These characteristics in combination with 16S rRNA gene sequence analysis revealed that the novel strains belonged to the genus *Demequina*. The DNA–DNA hybridization values showed that the three new strains belonged to the same species, a novel species of the genus *Demequina*. Therefore strains KV-810^T, KV-811 and KV-816 are proposed as representing a novel species, *Demequina salsinemoris* sp. nov. The type strain is KV-810^T (=DSM 22060^T=NBRC 105323^T).

Takahashi *et al.* (2003) reported that the apparent number of bacterial strains on an agar medium is increased by the addition of oxidant scavengers. Takahashi *et al.* (2006) proposed the genus *Patulibacter*, members of which were isolated using an agar medium supplemented with superoxide dismutase and which possessed demethylmenaquinone 7 (DMK-7) as the isoprenoid quinone. In this study, strains KV-810^T, KV-811 and KV-816 were isolated using agar media supplemented with ascorbic acid or rutin as free-radical scavengers. The strains contained DMK-9(H_4) as a diagnostic isoprenoid quinone. Actinobacterial species containing demethylmenaquinone are uncommon and it is known that the characteristic feature of the genus *Demequina* (Yi *et al.*, 2007) is the presence of demethylmenaquinone DMK-9(H_4). At present, the genus *Demequina* consists of two recognized species, *Demequina aestuarii* and *Demequina lutea*. In this study, the taxonomic characteristics of the isolated strains are reported and a novel species of the genus *Demequina* is described.

Strains KV-810^T, KV-811 and KV-816 were isolated from mangrove soil of Amami Island, which lies in the sea south of Kagoshima Prefecture, Japan. Soil (1 g) was diluted and spread on GPM plates (1 % glucose, 0.5 % peptone, 0.5 % meat extract, 0.3 % NaCl, 1.2 % agar; pH 7.0) containing Benlate (20 mg l^{-1}; DuPont) with ascorbic acid (440 μg l^{-1}) or rutin (120 μg l^{-1}), which are known to be free-radical scavengers. After cultivation at 27 °C, strains KV-810^T and KV-811 were isolated from GPM plates supplemented with ascorbic acid and strain KV-816 was isolated from GPM plates supplemented with rutin.

Morphological characteristics were observed by scanning electron microscopy (JSM 5600; JEOL) following incubation on TSY agar (3 % Bacto tryptic soy broth, 0.3 % yeast extract, 1.2 % agar) for 3 days at 27 °C and fixation by 4 % osmium tetroxide vapour. API ZYM, API 20NE and API 50CH tests (bioMérieux) were used for biochemical characterization, according to the manufacturer's instructions. API ZYM and API 20NE tests were performed at 27 °C for 24 h and API 50CH was incubated at 72 h (except that the cultivation of *D. lutea* SV45^T was 20 °C for 1 week). Hydrolysis of casein and starch and decomposition of adenine, hypoxanthine, L-tyrosine and xanthine were examined on TSY agar according to Gordon *et al.* (1974) after incubation for 2 weeks at 27 or 20 °C. YD agar (1.0 % yeast extract, 1.0 % glucose, 1.2 % agar; pH 7.0) was used for the NaCl tolerance test. TSY agar was used for the pH and temperature range tests. DNase test agar (Difco) was used for the DNase assay. Triple-sugar iron agar (Difco) was used to test for H_2S production. Results were recorded after cultivation for 2 weeks.

Biomass for chemotaxonomy was prepared by cultivation in TSY broth (3 % Bacto tryptic soy broth, 0.3 % yeast extract) for 5 days at 27 °C. *N*-Acyl types of muramic acid were determined by the method of Uchida & Aida (1977). Purified cell-wall extracts were obtained using the method of Kawamoto *et al.* (1981) and the amino acid composition of hydrolysed cell walls was determined by HPLC using the

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KV-810^T, KV-811 and KV-816 are AB471559, AB471560 and AB471561, respectively.

Scanning electron micrographs of cells of strains KV-810^T and KV-816 and a supplementary table detailing the DNA–DNA relatedness values between strains KV-810^T, KV-811 and KV-816 and *Demequina aestuarii* JC2054^T are available with the online version of this paper.

Pico-Tag method (Waters). The presence of mycolic acids was examined by TLC according to Tomiyasu (1982) and phospholipids were extracted and identified following the method of Minnikin *et al.* (1977). Menaquinones were extracted and purified by the method of Collins *et al.* (1977) and were subsequently analysed by LCMS (JMS T100LP; JEOL) with a Pegasil ODS column (20 × 50 mm) using methanol/2-propanol (7 : 3). Biomass for analysis of fatty acids was prepared by culturing on TSY agar for 5 days at 27 °C and analysis was performed according to the procedures for the Sherlock Microbial Identification System (Microbial ID).

Chromosomal DNA was isolated as described by Saito & Miura (1963), with some modifications. DNA base composition was estimated by the HPLC method of Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were performed as described by Ezaki *et al.* (1989). 16S rRNA gene sequences were obtained using previously described methods (Matsumoto *et al.*, 2008). CLUSTAL_X version 1.83 (Thompson *et al.*, 1994) was used for multiple alignments with selected sequences and for calculating evolutionary distances (Kimura, 1980). A phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987). Data were resampled with 1000 bootstrap replications (Felsenstein, 1985). For the creation of a phylogenetic tree by the maximum-likelihood method (Felsenstein, 1981), PHYLIP version 3.67 was used. Sequence similarity values were determined by visual comparison and manual calculation.

The cell-wall peptidoglycan of strains KV-810^T, KV-811 and KV-816 contained L-ornithine as the diamino acid and alanine, serine and glutamic acid. The acyl type of the peptidoglycan was acetyl. The predominant menaquinone was DMK-9(H_4). Mycolic acids were not detected. Only unidentified phospholipids were detected and phosphatidylinositol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine were absent. The G+C content of the genomic DNA was 70–72 mol%.

Almost-complete 16S rRNA gene sequences for strains KV-810^T (1436 bp), KV-811 (1395 bp) and KV-816 (1386 bp) were determined. The sequence similarity between the three strains was 100.0 %, indicating that they belonged to the same species. Phylogenetic analysis with the neighbour-joining and the maximum-likelihood methods showed that these strains were closely related to the genus *Demequina* of the suborder *Micrococcineae* (Fig. 1). Although the phylogenetic and chemotaxonomic data clearly showed that the strains belonged to the genus *Demequina*, the 16S rRNA gene sequence similarity values between the new isolates and *D. aestuarii* JC2054^T and *D. lutea* SV45^T were low (96.9 and 95.5 %, respectively).

Strains KV-810^T, KV-811 and KV-816 grew well on TSY agar, TSB agar and YD agar at 27 °C and colonies were pale yellow. Cells were non-motile and rods (0.3–0.4 × 0.7–1.1 μm) (see Supplementary Fig. S1, available in IJSEM Online).

The physiological differences between strains KV-810^T, KV-811 and KV-816, *D. aestuarii* JC2054^T and *D. lutea* SV45^T are shown in Table 1. All three isolates showed some differences from the recognized species of the genus *Demequina*. The range of sodium chloride concentration for growth of the isolates was 0–8 % (w/v); *D. aestuarii* was strongly tolerant (12 %) and *D. lutea* was sensitive (2 %). Strain KV-810^T and the type strains of the two recognized

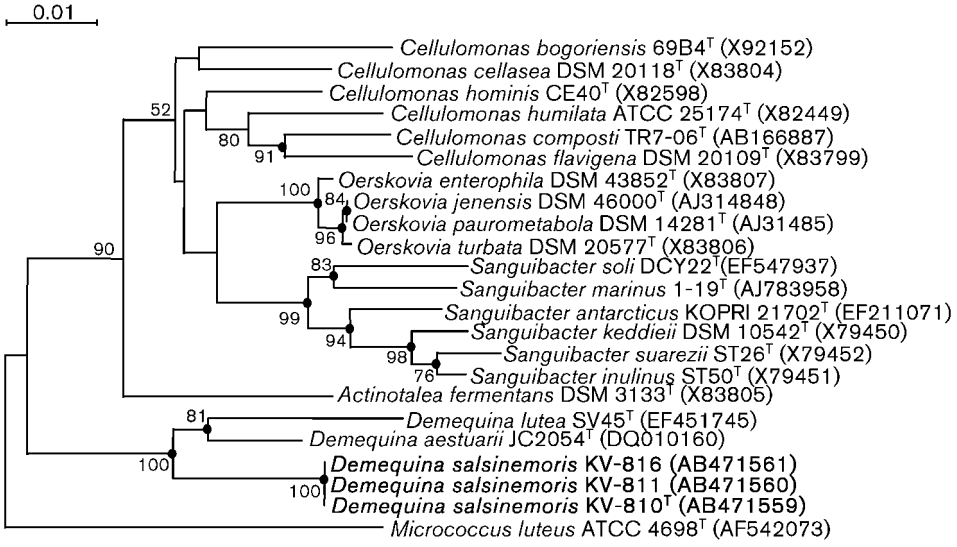

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences and constructed using the neighbour-joining method. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. Bar, 1 substitution per nucleotide position.

Table 1. Differential phenotypic characteristics of strains KV-810^T, KV-811 and KV-816 and the type strains of species of the genus *Demequina*

Strains: 1, KV-810^T; 2, KV-811; 3, KV-816; 4, *D. aestuarii* JC2054^T; 5, *D. lutea* SV45^T. +, Positive; w, weakly positive; −, negative.

Characteristic	1	2	3	4	5
Temperature range for growth (°C)	14–34	12–34	12–34	5–35*	4–30†
pH range for growth	6–9	6–9	5–9	6–11*	6–9†
Tolerance to NaCl (%)	8	8	8	12*	2†
API ZYM					
Acid phosphatase	w	w	w	−	+
Alkaline phosphatase	−	−	−	w	−
Esterase lipase (C8)	w	w	w	−	−
Naphthol-AS-BI-phosphohydrolase	w	w	w	w	−
α-Galactosidase	w	w	w	−	+
α-Glucosidase	+	+	+	−	+
API 50CH					
D-Arabinose	−	−	−	+	−
D-Galactose	+	+	+	−	w
Gentiobiose	+	+	+	+	−
Maltose	+	+	+	−	+
L-Rhamnose	+	+	+	−	−
Sucrose	−	−	−	+	+
Trehalose	−	−	−	−	+
Amygdalin	+	+	+	+	−
Arbutin	+	+	+	−	−
Methyl β-D-xylopyranoside	+	+	+	−	w

*Data from Yi *et al.* (2007).
†Data from Finster *et al.* (2009).

species of the genus *Demequina* possessed anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{16:0}$ as the dominant cellular fatty acids (Table 2).

Among the three new isolates, the DNA–DNA hybridization values were 74–99 %. The DNA–DNA hybridization values were very low (4–9 %) between the new isolates and *D. aestuarii* JC2054^T (see Supplementary Table S1 in IJSEM Online) and were below the 70 % cut-off value recommended for the delineation of species (Wayne *et al.*, 1987). On the basis of these results and the chemotaxonomic and phylogenetic analyses, we propose that strains KV-810^T, KV-811 and KV-816 represent a novel species of the genus *Demequina*, *Demequina salsinemoris* sp. nov.

Table 2. Cellular fatty acid contents (%) of strain KV-810^T, *D. aestuarii* JC2054^T and *D. lutea* SV45^T

Strains: 1, KV-810^T; 2, *D. aestuarii* JC2054^T; 3, *D. lutea* SV45^T. Data were taken from this study, Yi *et al.* (2007) and Finster *et al.* (2009). Fatty acids that represented <1 % are omitted.

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	5.8	1.6	1.8
$C_{15:0}$	1.6	4.2	3.2
$C_{16:0}$	15.6	10.4	1.4
Branched			
iso-$C_{14:0}$	1.7	1.1	2.3
iso-$C_{15:0}$	1.6	3.3	3.1
anteiso-$C_{15:0}$	45.7	46.8	59.4
anteiso-$C_{15:1}$	1.5	10.2	6.4
iso-$C_{16:0}$	6.3	7.0	8.9
anteiso-$C_{17:0}$	16.1	13.1	8.8

Description of *Demequina salsinemoris* sp. nov.

Demequina salsinemoris (sal.si.ne.mo′ris. L. adj. *salsus* salted; L. n. *nemus -oris* wood; N.L. gen. n. *salsinemoris* of a salted wood, isolated from soil of a salted wood, a mangrove).

Cells are rods and 0.3–0.4 × 0.7–1.1 μm in size. Aerobic. The temperature range for growth is 12–34 °C. Growth occurs at initial pH values of between 5 and 9. NaCl is tolerated, up to 8 % (w/v), but is not required. Good growth occurs on TSB agar and YD agar. Colonies are pale yellow. Catalase-positive and oxidase- and DNase-negative. H_2S is not produced. With API 20NE, hydrolysis of aesculin and β-galactosidase are positive, but nitrate reduction, indole production and acid production from glucose, arginine dihydrolase, urease and gelatin are negative. With API ZYM, acid phosphatase, esterase

(C4), esterase lipase (C8), α- and β-galactosidases, α- and β-glucosidases, leucine arylamidase and naphthol-AS-BI-phosphohydrolase are present, but *N*-acetyl-β-glucosaminidase, alkaline phosphatase, chymotrypsin, cystine arylamidase, α-fucosidase, β-glucuronidase, lipase (C4), α-mannosidase and trypsin and valine arylamidases are absent. With API 50CH, aesculin, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, maltose, D-mannose, methyl β-D-xylopyranoside, L-rhamnose, salicin and D-xylose are utilized as carbon sources but *N*-acetyl-glucosamine, D-adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, glycerol, glycogen, inositol, inulin, 2- and 5-ketogluconate, D-lactose, D-lyxose, D-mannitol, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, gluconate, raffinose, D-ribose, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose and xylitol are not utilized. Hydrolysis of casein and starch and decomposition of adenine, hypoxanthine, L-tyrosine and xanthine are negative. The predominant components of the cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{16:0}$.

The type strain, KV-810^T (=DSM 22060^T=NBRC 105323^T), was isolated from mangrove soil of a southern island in Japan. The DNA G+C content of the type strain is 70–72 mol%.

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