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Desulfoplanes formicivorans gen. nov., sp. nov., a novel sulfate-reducing bacterium isolated from a blackish meromictic lake, and emended description of the family *Desulfomicrobiaceae*

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Running head: *Desulfoplanes formicivorans* gen. nov., sp. nov.

Subject category: New taxa: *Proteobacteria*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain Pf12B^T is LC017841. The accession number for *dsrA* gene is LC017842. The accession number for *aprA* gene is LC017843.

Abstract

A novel sulfate-reducing bacterium, designated Pf12B^T, was isolated from sediment of a meromictic lake in Japan, Lake Harutori. Cells were vibroid (1.0× 3.0-4.0 μm), motile, and Gram-staining-negative. For growth, the optimum pH was 7.0-7.5 and the optimum temperature was 42-45ºC. Strain Pf12B^T used sulfate, thiosulfate, and sulfite as electron acceptors. The G+C content of the genomic DNA was 55.4 mol%. Major cellular fatty acids were $C_{16:0}$ and $C_{18:0}$. The strain was desulfoviridin positive. Phylogenetic analysis based on the 16S rRNA gene revealed that the novel strain belonged to the order *Desulfovibrionales* in the class *Deltaproteobacteria*. The closest relative was *Desulfomicrobium baculatum* DSM 4028^T with the 16S rRNA gene sequence similarity of 91%. On the basis of phylogenetic and phenotypic characterization, *Desulfoplanes formicivorans*, gen. nov. sp. nov., belonging to the family *Desulfomicrobiaceae* is proposed with the type strain Pf12B^T (=NBRC 110391^T =DSM 28890^T).

38 The order *Desulfovibrionales* was proposed in 2006 with the type genus *Desulfovibrio* by Kuever et al.
39 2006 (Validation List No. 107). Members of this order are strict anaerobes, and all of them are sulfate reducers
40 except for the genera *Lawsonia* and *Bilophila* (Kuever et al., 2009). The order is currently organized in four
41 families; *Desulfovibrionaceae*, *Desulfonatronumaceae*, *Desulfohalobiaceae* and *Desulfomicrobiaceae*. The
42 family *Desulfomicrobiaceae* is presently constituted by only one genus, *Desulfomicrobium*. The genus
43 *Desulfomicrobium* has been characterized by rod- or ellipsoidal-shaped morphology and absence of
44 desulfoviridin (Genthner & Devereux, 2009). In this study, we report on a novel bacterium related to these
45 organisms, which possesses desulfoviridin and vibroid morphology.
46 The strain Pf12B^T was isolated from sediment of Lake Harutori, situated in Kushiro, Hokkaido,
47 north-eastern Japan. It is a meromictic lake containing high concentration of H_2S in hypoxia (Kubo et al.,
48 2014). Lake sediment was collected at the deepest site (5.5 m) by a core sampler. From the sediment core,
49 12-15 cm layer (depth from sediment surface) was retrieved and then homogenized in a sealed plastic bag. To
50 establish the first enrichment culture, approx. 1 ml of the sediment slurry was inoculated into 40 ml of
51 bicarbonate-buffered sulfide-reduced defined basal medium containing sulfate (Widdel & Bak, 1992). One
52 milliliter of *p*-xylene solution (2% [vol・vol^{-1}] in 2,2,4,4,6,8-heptamethylnonane, which served as the carrier
53 phase [Rabus et al. 1993]) was supplemented to the medium as the sole carbon source and electron donor.
54 Headspace of the bottle was filled with N_2/CO_2 (80:20 [vol・vol^{-1}]), and incubation was carried out in the dark
55 at 45°C. Grown culture (1-2% volume of fresh medium) was transferred to the same medium. After three
56 transfers, *p*-xylene solution was replaced with formate (final concentration of 10 mM). The resulting culture
57 was transferred to the fresh medium twice, and then subjected to dilution in anoxic agar tubes (Widdel & Bak,
58 1992). Further, the agar tube dilution was repeated twice, utilizing the medium supplemented with 10 mM
59 formate and 0.5 g l^{-1} yeast extract. Finally, the pure culture termed Pf12B^T was obtained. Culture purity was
60 ascertained routinely by microscopy and checked by denaturing gradient gel electrophoresis of the 16S rRNA
61 gene (Muyzer et al. 1996) for cultures from physiological tests.
62 Cell morphology was confirmed by phase-contrast microscopy (Axioplan 2; Zeiss). The Gram-staining test

was carried out by using Gram-staining kit (Fluka). Presence of desulfoviridin was assessed by its red fluorescence under irradiation with UV light. Cells grown with 10 mM formate and 0.5 g l^{-1} yeast extract were collected by centrifugation, suspended in distilled water, and then disrupted by addition of NaOH (Engelkrik et al., 1992). The resulting lysate was immediately inspected under UV illumination. *Desulfovibrio piger* DSM 749^T and *Desulfomicrobium norvegicum* DSM 1741^T were used as positive and negative controls, respectively. The DNA G+C content of the strain was determined by using a Yamasa GC kit (Yamasa shoyu) with the HPLC methods as described previously (Katayama-Fujimura et al., 1984). Cellular fatty acids were analyzed by the identification services of Techno Suruga Laboratory Co., Ltd, using the Sherlock Microbial Identification System (MIDI) (Sherlock Version 6.0; MIDI database TSBA40). The biomass from cultures grown at 45ºC with 10 mM formate and 10 mM acetate was used for the analysis.

For physiological characterization of strain Pf12B^T, the basal medium containing 10 mM formate and 0.5 g l^{-1} yeast extract was used unless otherwise specified, and cultures were incubated at 45ºC. Utilization of electron donors was tested in the media each containing one of the substrates listed later, and evaluated by monitoring the growth. Ability for growth with H_2-dependent sulfate reduction was assessed under a gas mixture ($H_2/N_2/CO_2$, 50:40:10; 2 atm total pressure), in the presence or absence of acetate (10 mM). Fermentative growth was tested with the media containing no sulfate, and supplemented with glucose, fumarate, malate, or pyruvate. Utilization of electron acceptors was tested using the sulfate-free basal media each containing one of the substances listed later. Range of NaCl concentrations for growth was tested at 10 different concentrations (0, 0.5, 1, 2, 4, 5, 6, 8, 9 and 10% [w/v]) by using the basal medium with lowered concentration of $MgCl_2$ (0.4 g l^{-1}). Growth at different temperatures was examined by incubation at 12 different temperatures (8, 13, 15, 18, 28, 32, 37, 42, 45, 50, 55 and 58 ºC). In order to determine the pH range for growth of Pf12B^T, $NaHCO_3$ in the basal medium was replaced with 10 g l^{-1} of MES, MOPS or CHES. The pH of the medium was adjusted with HCl or NaOH, and growth was tested at 10 different pH values (5.4, 6.1, 7.0, 7.3, 7.5, 7.9, 8.1, 8.6, 9.0 and 9.4).

For phylogenetic analysis, genomic DNA of the strain was purified with Wizard Genomic DNA Purification

Kit (Promega). The 16S rRNA gene fragments were amplified with primers 27f and 1492r (Lane, 1991). Fragments of the *aprA* gene encoding adenosine-5′-phosphosulfate reductase were amplified with a primer set of Apr-1-FW/Apr-5-RV (Meyer & Kuever, 2007). Fragments of the gene for alpha subunit of dissimilatory sulfite reductase (*dsrA*) were amplified with primers DSR1Fdeg (Klein et al., 2001) and DSR1334R (Santillano et al., 2010). PCR amplification was carried out using Takara Ex Taq DNA polymerase (Takara), and PCR products were directly sequenced. Nucleotide sequences of the PCR products were determined by cycle sequencing with a dye terminator (BigDye® Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems). The obtained nucleotide sequence of the 16S rRNA gene and amino acid sequences deduced from the *dsrA* and *aprA* genes were aligned with reference sequences from public databases (GenBank/EMBL/DDBJ) using the ClustalX version 2.1 program (Larkin et al. 2007). Phylogenetic trees were constructed by the Maximum-Likelihood method with the program *MEGA* version 5.1 (Tamura *et al*., 2011). Bootstrap analysis was performed for 1000 replicates.

Cells of strain Pf12B^T were vibroid, (1.0 × 3.0-4.0 μm), motile, Gram-staining-negative, and occur singly or in pairs or chains (Fig. 1). Endospores were not observed. The G+C content of genomic DNA was 55.4 mol%. Total cellular fatty acid profile of strain Pf12B^T is summarized in Table 1. Major cellular fatty acids were $C_{16:0}$ (41.1%) and $C_{18:0}$ (20.8%). Cell lysate of strain Pf12B^T exhibited red fluorescence, suggesting the presence of desulfoviridin.

The growth temperature and pH are shown in Table 2. Growth of the strain Pf12B^T was observed in the media containing 0.5-8% of NaCl, and the optimal concentration for growth was 1-4%. Under sulfate-reducing conditions, the strain grew on following substrates (mM, except where stated): fumarate (10), formate (10), lactate (10), and H_2 (partial pressure of 1 atm) in the presence of acetate. Autotrophic growth with H_2 was not observed. Addition of yeast extract (0.5 g l^{-1}) greatly enhanced the growth on formate. In comparison to the other substrates, growth on lactate was exceedingly slow. Slight fermentative growth was observed with pyruvate (10), malate (10) and fumarate (10), but not with glucose (10). The following

113 substrates could not support growth of the strain under sulfate-reducing conditions: acetate (10), succinate
114 (10), propionate (5), benzoate (2.5), ethanol (10), glucose (10) and yeast extract (0.5 g l^{-1}). The strain used
115 sulfate (28), thiosulfate (10) and sulfite (5) as an electron acceptor to support the growth, but not nitrate (10).
116 Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain Pf12B^T is a member of the
117 order *Desulfovibrionales* in the class *Deltaproteobacteria* (Fig. 2). It was also shown that strain Pf12B^T
118 belongs to the family *Desulfomicrobiaceae*, with high bootstrap value support (97%). The closest cultivated
119 relative of strain Pf12B^T was *Desulfomicrobium baculatum* (91% sequence similarity). The most closely
120 related environmental clones were TCB4y (Dahle et al., 2008) and SBYG 5325 (Harris et al., 2013) with 99%
121 and 96% sequence similarities, respectively. In the phylogenetic trees based on DsrA and AprA amino acid
122 sequences, strain Pf12B^T was placed in a cluster of the order *Desulfovibrionales* (Fig. 3, Fig .S1).
123 As shown in Table 1 and Table 2, there were distinct phenotypic differences between Pf12B^T and members
124 of the genus *Desulfomicrobium*. All known *Desulfomicrobium* species have rod- or ellipsoidal-shaped
125 morphology (Genthner, 2009), but strain Pf12B^T was vibroid (Fig. 1, Table 2). Although desulfoviridin is
126 absent from *Desulfomicrobium* species, it was detected from strain Pf12B^T. In contrast to typical
127 *Desulfomicrobium* species, strain Pf12B^T required NaCl for growth and could not use ethanol as growth
128 substrate. The strain Pf12B^T was characterized by high concentration of saturated fatty acids, as well as
129 absence of fatty acids prevalent in the *Desulfomicrobium* species, iso-$C_{17:1}$ and $C_{18:1\omega11c}$ (Table 1).
130 On the basis of these results, we propose that strain Pf12B^T represents a novel species of a novel genus
131 within the family *Desulfomicrobiaceae*. We propose the name *Desulfoplanes formicivorans* gen. nov., sp. nov.
132 with the type strain Pf12B^T (=NBRC 110391^T =DSM 28890^T).
133
134
135 **Emended description of the family *Desulfomicrobiaceae***
136 All known members are mesophilic or thermophilic sulfate-reducing bacteria. Organic substrates are
137 incompletely oxidized to acetate. The family comprises two genera; *Desulfomicrobium* and *Desulfoplanes*.

Type genus is *Desulfomicrobium*.

Description of *Desulfoplanes* gen. nov.

Desulfoplanes (De.sul.fo. plan'es. L. prefix *de* off; L. n. *sulfur* sulfur; Gr. n. *planes*, a wanderer, roamer; N.L. masc. n. *Desulfoplanes*, a sulfate-reducing wanderer; pertaining to a motile sulfate reducer). Gram-negative, strictly anaerobic. Endospores not formed. Sulfate is used as electron acceptors. Desulfoviridin positive. NaCl is required for growth. Saturated fatty acids are major components in cellular fatty acid profile. Phylogenetically, the genus *Desulfoplanes* belongs to the order *Desulfovibrionales* in the class *Deltaproteobacteria*. The type species is *Desulfoplanes formicivorans*.

Description of *Desulfoplanes formicivorans* gen. nov., sp. nov.

Desulfoplanes formicivorans (for.mi.ci.vo'rans. N.L. n. *acidum formicum*, formic acid; L. part. adj. *vorans*, eating; N.L. adj. *formicivorans*, eating formic acid). Cells are vibroid, 1.0 × 3.0-4.0 μm, motile and occurring singly, in pairs or in chains. The temperature range for growth was 13-50°C, with an optimum temperature range at 42-45°C. The pH range for growth is 6.1-8.6, with a range of optimum growth at pH 7.0-7.5. The NaCl concentration for growth is 0.5-8%. The optimal concentration of NaCl for growth was 1-4%. In addition to sulfate, thiosulfate and sulfite are used as electron acceptors for growth. Nitrate is not used as electron acceptor. Formate, fumarate, lactate, and H_2 are utilized as electron donors in the presence of sulfate. Acetate, succinate, propionate, benzoate, ethanol are not utilized as electron donor. Pyruvate, malate and fumarate are also used for fermentative growth. The major cellular fatty acids are $C_{16:0}$ and $C_{18:0}$. The DNA G+C content of the type strain is 55.4 mol%.

The type strain, Pf12B^T (=NBRC 110391^T =DSM 28890^T), was isolated from sediment of a meromictic lake in Japan (Lake Harutori).

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Figure legends

Fig. 1 Phase-contrast micrograph of strain Pf12B^T grown with formate (10 mM) and yeast extract (0.5 g l^{-1}) in the presence of sulfate for 2 days. Bar, 10 μm.

Fig. 2 Maximum-likelihood tree based on 16S rRNA gene sequence of strain Pf12B^T and the members of the order *Desulfovibrionales*. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes.

Fig. 3 Maximum-likelihood tree showing the phylogenetic position of strain Pf12B^T within the order *Desulfovibrionales*, based on DsrA amino acid sequences. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes.

Table 1. Cellular fatty acid content (percentage of total) of strain Pf12B^T and other members of the family *Desulfomicrobiaceae*. Taxa: 1, *Desulfoplanes formicivorans* Pf12B^T; 2, *D. baculatum* (four strains); 3, *D. orale* NY678^T; 4, *D. apsheronum* DSM 5918^T; 5, *D. norvegicum* DSM 1741^T. Data of *Desulfomicrobium* species were obtained from Langendijk et al. Abbreviations: c, cis; cyclo, cyclopropyl; dma, dimethylacetal; F, H, different positions of double bonds; OH, hydroxyl. Undefined fatty acids were also observed in the fatty acid profile of Pf12B^T, summed feature 2 ($C_{12:0}$ aldehyde, unknown 10.928, iso-$C_{16:1}$ I and $C_{14:0}$ 3OH; 0.9% of the total), 3 ($C1_{6:1}\omega7c$ and $C_{16:1}\omega6c$; 0.8%), 7 (unknown 18.846, $C_{19:1}\omega6c$ and $C_{19:0}$ cyclo$\omega10c$; 1.0%), 8 ($C_{18:1}\omega7c$ and $C_{18:1}\omega6c$; 3.0%), 9 (iso-$C_{17:1}\omega9c$ and 10-methyl $C_{16:0}$; 0.6%).

Fatty acid	1	2	3	4	5
12:0		0–0.4		0–1.9	
12:0 2OH	0.2				
13:0					1.8
14:0 iso	1.0				
14:0	2.5	0–2.9	0.9	0.3–8.0	
15:1 iso F	0.2				
15:1 iso		1.5–4.0		0–2.4	3.6
15:0 iso	6.0	4.7–11.6	12.4	3.0–7.1	11.6
15:0 anteiso	4.7	3.5–7.7	5.6	2.0–4.7	7.0
15:0		0–0.8	0.6	0–3.7	
16:1 iso F		0–0.9		0–1.0	
16:1 iso H		0–2.1		0–2.8	0.9
15:0 dma		0–1.2	1.3		
16:0 iso	4.3	0–1.0	1.0	0–1.3	
16:1 c9	0.3	2.2–9.4	6.7	6.2–8.3	2.4
16:1 c11			0.8	0–0.4	
16:0	41.1	3.6–15.7	18.8	4.3–42	2.4
15:0 iso 3OH	0.9	1.2–2.4	1.9	0.2–1.8	3.8
15:0 anteiso 3OH		0–1.2	0.5	0–0.7	1.7
15:0 2OH	0.4				
17:1 iso		4.0–28.6	6.7	1.3–17.8	28.8
16:0 dma		0–0.6		0–0.3	0.6
17:1 anteiso		0–4.1	1.8	0–2.6	4.1
17:1 anteiso c9	0.2				
17:0 iso	0.8	0.9–6.9	3.2	1.2–3.6	6.3
17:0 anteiso	1.7	2.0–8.3	2.3	1.7–4.1	6.7
17:1 c9			1.1	0–0.4	
17:1 c11			1.2	0–0.2	
17:0 cyclo	0.8		3.7		
17:0	1.4	0.2–0.9		0.4–1.9	
16:0 3OH	1.9	0–0.9	1.7	0–0.8	0.4
18:1 c9	1.5	2.6–6.3	2.4	2.4–6.3	2.2
18:1 c11		6.4–14.2	7.9	1.0–17.6	5.9
18:0	20.6	6.0–22.8	16.8	9.0–19.2	2.7
17:0 iso 3OH		0–2.2		0–0.7	2.2
18:0 dma		0–0.8		0–0.8	
19:0 cyclo c8	1.9				
18:0 3OH	1.1	0–0.9	0.8		0.3
20:0		0–0.4		0–1.1	

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Table 2. Characteristics of strain Pf12B^T and members of the genus *Desulfomicrobium*. Taxa: 1, *Desulfoplanes formicivorans* Pf12B^T; 2, *D. baculatum*; 3, *D. orale*; 4, *D. escambiense*; 5, *D. salsuginis*; 6, *D. thermophilum*; 7, *D. apsheronum*; 8, *D. norvegicum*. Data were obtained from Dias et al. (2008), Thevenieau et al. (2007), and Genthner & Devereux (2009). +, growth; –, no growth; (+), slight growth; nr, not reported.

	1	2	3	4	5	6	7	8
Morphology	Vibroid	Rod	Rod	Rod	Rod	Rod	Rod	Rod
DNA G+C content (mol%)	55.4	56.8	59.7	59.9	63.1	58.7	52.5	56.3
Desulfoviridin	+	–	–	–	–	–	–	–
Optimum temperature (°C)	42–45	28–37	37	25–30	35	55	25–30	25–30
NaCl requirement	+	–	nr	–	–	–	–	–
Electron acceptors								
Sulfate	+	+	+	+	+	+	+	+
Sulfite	+	+	nr	nr		+	+	+
Thiosulfate	+	+	nr	+	+	+	+	+
Nitrate	–	–	nr	–	+	–	–	nr
Electron donors								
Ethanol	–	+	+	+	+	+	+	+
Fermentation								
Pyruvate	(+)	(+)	(+)	+	(+)	+	(+)	(+)
Malate	(+)	+	nr	+	(+)	+	+	+
Fumarate	(+)	(+)	nr	+	+	(+)	(+)	+