



# HOKKAIDO UNIVERSITY

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| Title            | Desulfoplanes formicivorans gen. nov., sp nov., a novel sulfate-reducing bacterium isolated from a blackish meromictic lake, and emended description of the family Desulfomicrobiaceae                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| Author(s)        | Watanabe, Miho; Kojima, Hisaya; Fukui, Manabu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| Citation         | International journal of systematic and evolutionary microbiology, 65, 1902-1907<br><a href="https://doi.org/10.1099/ijs.0.000197">https://doi.org/10.1099/ijs.0.000197</a>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Issue Date       | 2015-06-01                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Doc URL          | <a href="https://hdl.handle.net/2115/61993">https://hdl.handle.net/2115/61993</a>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
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| Type             | journal article                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| File Information | manuscript_150313.pdf                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |



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

Miho Watanabe, Hisaya Kojima, Manabu Fukui

The Institute of Low Temperature Science, Hokkaido University, Sapporo, Hokkaido, Japan

Correspondence: Miho Watanabe, Institute of Low Temperature Science, Hokkaido University,  
Nishi 8, Kita 19, Kita-ku Sapporo, Hokkaido 060-0819, Japan.  
Tel.: +81 11 706 5460; fax: +81 11 706 5460; e-mail: m.watanabe@pop.lowtem.hokudai.ac.jp

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<sup>T</sup> is  
LC017841. The accession number for *dsrA* gene is LC017842. The accession number for *aprA* gene is  
LC017843.

26   **Abstract**

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

37

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*, *Desulfonatrumaceae*, *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<sup>T</sup> 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<sub>2</sub>S 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<sup>-1</sup>] 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<sub>2</sub>/CO<sub>2</sub> (80:20 [vol · vol<sup>-1</sup>]), 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<sup>-1</sup> yeast extract. Finally, the pure culture termed Pf12B<sup>T</sup> 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

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

73 For physiological characterization of strain Pf12B<sup>T</sup>, the basal medium containing 10 mM formate and 0.5 g  
74 l<sup>-1</sup> yeast extract was used unless otherwise specified, and cultures were incubated at 45°C. Utilization of  
75 electron donors was tested in the media each containing one of the substrates listed later, and evaluated by  
76 monitoring the growth. Ability for growth with H<sub>2</sub>-dependent sulfate reduction was assessed under a gas  
77 mixture (H<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub>, 50:40:10; 2 atm total pressure), in the presence or absence of acetate (10 mM).  
78 Fermentative growth was tested with the media containing no sulfate, and supplemented with glucose,  
79 fumarate, malate, or pyruvate. Utilization of electron acceptors was tested using the sulfate-free basal media  
80 each containing one of the substances listed later. Range of NaCl concentrations for growth was tested at 10  
81 different concentrations (0, 0.5, 1, 2, 4, 5, 6, 8, 9 and 10% [w/v]) by using the basal medium with lowered  
82 concentration of MgCl<sub>2</sub> (0.4 g l<sup>-1</sup>). Growth at different temperatures was examined by incubation at 12  
83 different temperatures (8, 13, 15, 18, 28, 32, 37, 42, 45, 50, 55 and 58 °C). In order to determine the pH range  
84 for growth of Pf12B<sup>T</sup>, NaHCO<sub>3</sub> in the basal medium was replaced with 10 g l<sup>-1</sup> of MES, MOPS or CHES. The  
85 pH of the medium was adjusted with HCl or NaOH, and growth was tested at 10 different pH values (5.4, 6.1,  
86 7.0, 7.3, 7.5, 7.9, 8.1, 8.6, 9.0 and 9.4).

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

88 Kit (Promega). The 16S rRNA gene fragments were amplified with primers 27f and 1492r (Lane, 1991).  
89 Fragments of the *aprA* gene encoding adenosine-5'-phosphosulfate reductase were amplified with a primer set  
90 of Apr-1-FW/Apr-5-RV (Meyer & Kuever, 2007). Fragments of the gene for alpha subunit of dissimilatory  
91 sulfite reductase (*dsrA*) were amplified with primers DSR1Fdeg (Klein et al., 2001) and DSR1334R  
92 (Santillano et al., 2010). PCR amplification was carried out using Takara Ex Taq DNA polymerase (Takara),  
93 and PCR products were directly sequenced. Nucleotide sequences of the PCR products were determined by  
94 cycle sequencing with a dye terminator (BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit; Applied  
95 Biosystems). The obtained nucleotide sequence of the 16S rRNA gene and amino acid sequences deduced  
96 from the *dsrA* and *aprA* genes were aligned with reference sequences from public databases  
97 (GenBank/EMBL/DDBJ) using the ClustalX version 2.1 program (Larkin et al. 2007). Phylogenetic trees  
98 were constructed by the Maximum-Likelihood method with the program *MEGA* version 5.1 (Tamura *et al.*,  
99 2011). Bootstrap analysis was performed for 1000 replicates.

100

101 Cells of strain Pf12B<sup>T</sup> were vibroid, (1.0 × 3.0-4.0 μm), motile, Gram-staining-negative, and occur singly or  
102 in pairs or chains (Fig. 1). Endospores were not observed. The G+C content of genomic DNA was 55.4 mol%.  
103 Total cellular fatty acid profile of strain Pf12B<sup>T</sup> is summarized in Table 1. Major cellular fatty acids were C<sub>16:0</sub>  
104 (41.1%) and C<sub>18:0</sub> (20.8%). Cell lysate of strain Pf12B<sup>T</sup> exhibited red fluorescence, suggesting the presence of  
105 desulfovibrin.

106 The growth temperature and pH are shown in Table 2. Growth of the strain Pf12B<sup>T</sup> was observed in the  
107 media containing 0.5-8% of NaCl, and the optimal concentration for growth was 1-4%. Under  
108 sulfate-reducing conditions, the strain grew on following substrates (mM, except where stated): fumarate (10),  
109 formate (10), lactate (10), and H<sub>2</sub> (partial pressure of 1 atm) in the presence of acetate. Autotrophic growth  
110 with H<sub>2</sub> was not observed. Addition of yeast extract (0.5 g l<sup>-1</sup>) greatly enhanced the growth on formate. In  
111 comparison to the other substrates, growth on lactate was exceedingly slow. Slight fermentative growth was  
112 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<sup>-1</sup>). 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<sup>T</sup> is a member of the  
117 order *Desulfovibrionales* in the class *Deltaproteobacteria* (Fig. 2). It was also shown that strain Pf12B<sup>T</sup>  
118 belongs to the family *Desulfomicrobiaceae*, with high bootstrap value support (97%). The closest cultivated  
119 relative of strain Pf12B<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> and members  
124 of the genus *Desulfomicrobium*. All known *Desulfomicrobium* species have rod- or ellipsoidal-shaped  
125 morphology (Genthner, 2009), but strain Pf12B<sup>T</sup> was vibroid (Fig. 1, Table 2). Although desulfoviridin is  
126 absent from *Desulfomicrobium* species, it was detected from strain Pf12B<sup>T</sup>. In contrast to typical  
127 *Desulfomicrobium* species, strain Pf12B<sup>T</sup> required NaCl for growth and could not use ethanol as growth  
128 substrate. The strain Pf12B<sup>T</sup> was characterized by high concentration of saturated fatty acids, as well as  
129 absence of fatty acids prevalent in the *Desulfomicrobium* species, iso-C<sub>17:1</sub> and C<sub>18:1ω11c</sub> (Table 1).

130 On the basis of these results, we propose that strain Pf12B<sup>T</sup> 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<sup>T</sup> (=NBRC 110391<sup>T</sup> =DSM 28890<sup>T</sup>).

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*.

138 Type genus is *Desulfomicrobium*.

139

140

141 **Description of *Desulfoplanes* gen. nov.**

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

148

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

150 *Desulfoplanes formicivorans* (for.mi.ci.vo'rans. N.L. n. *acidum formicum*, formic acid; L. part. adj. *vorans*,  
151 eating; N.L. adj. *formicivorans*, eating formic acid). Cells are vibroid, 1.0 × 3.0–4.0 µm, motile and occurring  
152 singly, in pairs or in chains. The temperature range for growth was 13–50°C, with an optimum temperature  
153 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  
154 NaCl concentration for growth is 0.5–8%. The optimal concentration of NaCl for growth was 1–4%. In  
155 addition to sulfate, thiosulfate and sulfite are used as electron acceptors for growth. Nitrate is not used as  
156 electron acceptor. Formate, fumarate, lactate, and H<sub>2</sub> are utilized as electron donors in the presence of sulfate.  
157 Acetate, succinate, propionate, benzoate, ethanol are not utilized as electron donor. Pyruvate, malate and  
158 fumarate are also used for fermentative growth. The major cellular fatty acids are C<sub>16:0</sub> and C<sub>18:0</sub>. The DNA  
159 G+C content of the type strain is 55.4 mol%.

160 The type strain, Pf12B<sup>T</sup> (=NBRC 110391<sup>T</sup> =DSM 28890<sup>T</sup>), was isolated from sediment of a meromictic lake  
161 in Japan (Lake Harutori).

162



163 **Acknowledgement**

164 This study was supported by JSPS KAKENHI Grant Number 22370005 to Fukui and Research Fellow of  
165 Japan Society for the Promotion Science to Watanabe.

166

167

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248

249 **Figure legends**

250

251 Fig. 1 Phase-contrast micrograph of strain Pf12B<sup>T</sup> grown with formate (10 mM) and yeast extract (0.5 g l<sup>-1</sup>) in  
252 the presence of sulfate for 2 days. Bar, 10 µm.

253

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

257

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

261

262 Table 1. Cellular fatty acid content (percentage of total) of strain Pf12B<sup>T</sup> and other members of the family  
 263 *Desulfomicrobiaceae*. Taxa: 1, *Desulfoplanes formicivorans* Pf12B<sup>T</sup>; 2, *D. baculatum* (four strains); 3, *D.*  
 264 *orale* NY678<sup>T</sup>; 4, *D. apsheronum* DSM 5918<sup>T</sup>; 5, *D. norvegicum* DSM 1741<sup>T</sup>. Data of *Desulfomicrobium*  
 265 species were obtained from Langendijk et al. Abbreviations: c, cis; cyclo, cyclopropyl; dma, dimethylacetal; F,  
 266 H, different positions of double bonds; OH, hydroxyl. Undefined fatty acids were also observed in the fatty  
 267 acid profile of Pf12B<sup>T</sup>, summed feature 2 (C<sub>12:0</sub> aldehyde, unknown 10.928, iso-C<sub>16:1</sub> I and C<sub>14:0</sub> 3OH; 0.9% of  
 268 the total), 3 (C<sub>16:1</sub> ω7c and C<sub>16:1</sub> ω6c; 0.8%), 7 (unknown 18.846, C<sub>19:1</sub> ω6c and C<sub>19:0</sub> cycloω10c; 1.0%), 8  
 269 (C<sub>18:1</sub> ω7c and C<sub>18:1</sub> ω6c; 3.0%), 9 (iso-C<sub>17:1</sub> ω9c and 10-methyl C<sub>16:0</sub>; 0.6%).

270

| 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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273 Table 2. Characteristics of strain Pf12B<sup>T</sup> and members of the genus *Desulfomicrobium*. Taxa: 1,  
 274 *Desulfoplanes formicivorans* Pf12B<sup>T</sup>; 2, *D. baculatum*; 3, *D. orale*; 4, *D. escambiense*; 5, *D. salsuginis*; 6, *D.*  
 275 *thermophilum*; 7, *D. apsheronum*; 8, *D. norvegicum*. Data were obtained from Dias et al. (2008), Thevenieau  
 276 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<br>(mol%)   | 55.4    | 56.8  | 59.7 | 59.9  | 63.1 | 58.7 | 52.5  | 56.3  |
| Desulfoviridin              | +       | –     | –    | –     | –    | –    | –     | –     |
| Optimum<br>temperature (°C) | 42–45   | 28–37 | 37   | 25–30 | 35   | 55   | 25–30 | 25–30 |
| NaCl requirement            | +       | –     | nr   | –     | –    | –    | –     | –     |
| <i>Electron acceptors</i>   |         |       |      |       |      |      |       |       |
| Sulfate                     | +       | +     | +    | +     | +    | +    | +     | +     |
| Sulfite                     | +       | +     | nr   | nr    |      | +    | +     | +     |
| Thiosulfate                 | +       | +     | nr   | +     | +    | +    | +     | +     |
| Nitrate                     | –       | –     | nr   | –     | +    | –    | –     | nr    |
| <i>Electron donors</i>      |         |       |      |       |      |      |       |       |
| Ethanol                     | –       | +     | +    | +     | +    | +    | +     | +     |
| <i>Fermentation</i>         |         |       |      |       |      |      |       |       |
| Pyruvate                    | (+)     | (+)   | (+)  | +     | (+)  | +    | (+)   | (+)   |
| Malate                      | (+)     | +     | nr   | +     | (+)  | +    | +     | +     |
| Fumarate                    | (+)     | (+)   | nr   | +     | +    | (+)  | (+)   | +     |

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