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***Desulfocucumis palustris* gen. nov., sp. nov., a mesophilic sulfate reducer
belonging to the *Desulfotomaculum* subcluster Ig**

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Running head: *Desulfocucumis palustris* gen. nov., sp.nov.

Subject category: New taxa: *Firmicutes* and related organisms

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NAW-5^T is
LC183909.

20 **Abstract**

21 A mesophilic, endospore-forming, sulfate-reducing bacterium, designated as strain NAW-5^T was isolated
22 from marsh soil. Cells of strain NAW-5^T were Gram-negative staining, curved rods that were motile. Strain
23 NAW-5^T grew at 18–48°C (optimum growth at 32–37 °C) and pH 5.8–8.4 (optimum growth at pH 6.2–7.3).
24 Electron donors utilized were various organic acids and H₂ which support autotrophic growth. Fermentative
25 growth occurred on carboxylic acids, but not on sugar. Sulfate, thiosulfate and elemental sulfur were used as
26 electron acceptors. The respiratory isoprenoid quinone was MK-7. The genomic DNA G+C content of this
27 strain was 46.6 mol%. Sequence analysis of the 16S rRNA gene showed that strain NAW-5^T was affiliated to
28 the family “Desulfotomaculaceae” but the strain shared very low sequence similarity with any representatives
29 of this family (≥89%). Strain NAW-5^T belongs to the *Desulfotomaculum* subcluster Ig which does not include
30 validly named species. On the basis of significant differences in the phylogenetic and phenotypic properties
31 between strain NAW-5^T and related species, strain NAW-5^T represents novel species of a novel genus for
32 which the name *Desulfocucumis palustris* gen. nov., sp. nov. is proposed. The type strain is NAW-5^T (=DSM
33 102911^T = NBRC 112242^T).

34 The genus *Desulfotomaculum* is a group of obligatory anaerobic, spore-forming sulfate-reducing bacteria
35 [1, 2]. Because of the high phylogenetic divergence, *Desulfotomaculum* species have been classified into
36 seven subclusters, designated as *Desulfotomaculum* subcluster Ia-Ig [3, 4 and 5]. Among these subclusters,
37 only the *Desulfotomaculum* subcluster Ig lacks representative species with a validly published name to date.
38 In this study, a novel sulfate-reducing bacterium belonging to the *Desulfotomaculum* subcluster Ig was
39 isolated and characterized.

40 Strain NAW-5^T was isolated from the soil of a marsh (43°04'08"N 141°31'11"E) in the Nopporo Forest Park,
41 situated in Sapporo, Hokkaido, Japan. To establish the first enrichment culture, approximately 1 ml of the
42 sediment slurry was inoculated into 40 ml of bicarbonate-buffered sulfide-reduced defined basal medium
43 containing sulfate [6]. Acetate (10 mM) was added to the medium as the sole organic carbon source. Headspace
44 of the bottle was filled with N₂ /CO₂ (80 : 20, v/v), and incubation was carried out in the dark at 28°C. Grown
45 culture (1–2 % volume of fresh medium) was transferred to the same medium. After three transfers, dilution in
46 anoxic agar tubes was performed using 10 mM acetate and 0.5 g l⁻¹ yeast extract as substrate. After colony
47 isolation from the agar tube dilution, a pure culture of strain NAW-5^T was obtained with the extinction dilution
48 method. The purity of culture was ascertained routinely by microscopy and verified by denaturing gradient gel
49 electrophoresis of the 16S rRNA gene [7] for cultures used to perform physiological tests.

50 Cell morphology was confirmed by phase-contrast microscopy (Axioplan 2; Zeiss). The Gram-stain test
51 was performed by using a Fluka Gram-stain kit. Flagellum staining was performed as described by Leifson
52 [8]. The genomic DNA G + C content of the strain was carried out by using a Yamasa GC kit (Yamasa
53 Shoyu) with the HPLC methods as described by Katayama-Fujimura et al. [9]. Analyses of cellular fatty
54 acids and respiratory quinone were carried out by the identification services of Techno Suruga Laboratory.
55 Cellular fatty acids were identified with the Sherlock Microbial Identification System (MIDI) (Sherlock

56 Version 6.0; MIDI database MOORE6), and respiratory quinones were analyzed using HPLC [10]. The
57 presence of desulfovirdin was tested by using fluorescence test described by Postgate [11]. Biomass from
58 cultures grown at 28°C with 5 mM acetate and 0.5 g l⁻¹ yeast extract was used for these analyses.

59 For physiological characterization, each test was performed in duplicate at 28°C except for tests of growth
60 temperature. The basal medium containing 10 mM acetate and 0.5 g l⁻¹ yeast extract was used unless
61 otherwise specified. Temperature range for growth was examined by incubation at 13 different temperatures
62 (13, 15, 18, 22, 25, 28, 32, 35, 37, 42, 45, 48 and 50 °C). To determine the pH range for growth, basal
63 medium was buffered with 20 mM of MES or TAPS instead of NaHCO₃. The pH of the medium was
64 adjusted with HCl or NaOH, and growth was tested at 10 different pH values (5.5, 5.8, 6.2, 6.5, 6.9, 7.3, 7.7,
65 8.3, 8.4 and 8.7). Range of NaCl concentrations for growth was tested at 6 different concentrations (0, 0.1,
66 0.6, 1.1, 2.1, 3.1 and 4.1 % [w/v]). Utilization of electron donors was tested in the media each containing one
67 of the substrates listed later, and evaluated by monitoring the sulfate reduction. Sulfate reduction was
68 evaluated based on production of sulfide quantified with the previously described method [12]. Ability for
69 autotrophic growth with H₂-dependent sulfate reduction was assessed under a gas mixture (H₂/N₂/CO₂,
70 50:40:10; 2 atm total pressure). Fermentative growth was tested with the media containing no sulfate.
71 Utilization of electron acceptors was tested using the sulfate-free basal media each containing one of the
72 substances listed later.

73 For phylogenetic analysis, genomic DNA of strain NAW-5^T was purified with a Wizard Genomic DNA
74 Purification Kit (Promega). The 16S rRNA gene fragments were amplified with primers 27f and 1492r [13].
75 PCR amplification was carried out using Takara Ex Taq DNA polymerase (Takara), and PCR products were
76 directly sequenced by using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The
77 obtained nucleotide sequence of the 16S rRNA gene (1442 bp) was aligned with 53 reference sequences from

78 public databases (GenBank/EMBL/DDBJ) using the program CLUSTAL X version 2.1 [14]. All positions
79 with gaps were excluded from the calculation, and 1138 positions were included in the final dataset.
80 Phylogenetic trees were reconstructed with the program MEGA version 7.0.20 [15].

81 Cells of strain NAW-5^T were Gram-negative staining, motile and curved or slightly spiral-shaped rods (1.0
82 × 3-70 μm) (Fig. S1). The colony color of this strain was pale brown on the anaerobic agar tube containing
83 basal medium. Peritrichous flagella were observed (Fig S2). Cells of this strain did not make clear swelling
84 sporangium. Spherical terminal spores were observed. The G+C content of genomic DNA was 46.6 mol%.
85 Major cellular fatty acids were iso-C15:0 (52.8 %), iso-C17:0 (12.9 %) and cyclopropane-C17:0 (10.0 %).
86 The other fatty acids detected were C16:0 DMA (4.4 %), C16:0 (2.9 %), C16:0ω7c (2.1 %), iso-C15:0 DMA
87 (1.5 %), C18:0 (1.5 %), iso-C16:0 (1.3 %), C18:1ω7c (1.3%), aldehyde-C16:0 (0.5 %), C14:0 (0.5 %),
88 iso-C13:0 (0.3 %), C16:1ω7c DMA (0.3 %), summed features 3 (iso-C15:0 aldehyde; 1.1%) and 10
89 (C18:1ω7c; 0.5%). Menaquinone-7 (MK-7) was detected as the sole respiratory quinone. Desulfovibrio was
90 absent.

91 The growth temperature and pH ranges for growth were 18-48 °C (optimum, 32-37 °C), pH 5.8-8.4
92 (optimum, 6.2-7.3). The NaCl range for growth was 0-2.1 % (optimum, 0%). Under sulfate-reducing
93 conditions, the strain grew on the following substrates (mM, except where stated): acetate (10), formate (10),
94 lactate (10), fumarate (5), propionate (5), n-butyrate (5), iso-butyrate (5), pyruvate (5), ethanol (10), methanol
95 (5), yeast extract (0.5 g l⁻¹) and benzoate (2). Addition of yeast extract greatly enhanced the growth on acetate,
96 but it was not required for growth on other substrates. Autotrophic growth with H₂ was observed.
97 Fermentative growth was observed with pyruvate (10), lactate (10) benzoate (5) and fumarate (5). Glucose
98 (10) could not support growth of the strain under sulfate-reducing conditions or fermentative conditions. In
99 the presence of 5 mM benzoate, the strain used sulfate (28), thiosulfate (10) and elemental sulfur as an

electron acceptor to support the growth, but not sulfite (5), nitrate (10) nitrite (5) and poorly crystalline Fe (III) oxide (20). The characteristics of strain NAW-5^T are listed in Table 1.

The 16S rRNA gene analysis revealed that strain NAW-5^T was member of the family “Desulfotomaculaceae” [5] (Fig. 1), although the strain had less than 89 % sequence similarity to any species of this group. The closest cultivated relatives of strain NAW-5^T were sulfate-reducing bacterium R-AcetonA170 (with 99 % of sequence similarity; the compared length was 1342 bp) [16] and *Desulfotomaculum* sp. Ox39 (with 93% of sequence similarity; 1437 bp) [17]. These two strains are not available from culture collections and have not been validly named. They are included in the *Desulfotomaculum* subcluster Ig which contains no species with a validly published name [3, 18]. In the phylogenetic tree based on 16S rRNA gene sequence, strain NAW-5^T and relatives belong to the *Desulfotomaculum* cluster Ig with 100% of bootstrap value support (Fig. 1).

As shown in Tables 1, there were distinct phenotypic differences between strain NAW-5^T and relatives. The novel isolate characterized by large cell size (3-70 µm) and twisted cell-shape. On the basis of these results, we proposed that the strain NAW-5^T represents a novel species of a novel genus. We propose the name *Desulfocucumis palustris* gen. nov., sp. nov. with the type strain NAW-5^T (=DSM 102911^T = NBRC 112242^T).

Description of *Desulfocucumis* gen. nov.

Desulfocucumis (De.sul.fo.cu'cu.mis. L. pref. *de* from; L. neut. n. *sulfur* sulfur; L. masc. n. *cucumis* cucumber; N.L. masc. n. *Desulfocucumis* a sulfate-reducing cucumber-shaped bacteria). Cells are spore-forming, strictly anaerobic and mesophilic. Sulfate serves as an electron acceptor. Respiratory quinone

122 is MK-7. Phylogenetic position based on 16S rRNA gene is located within the family "*Desulfotomaculaceae*"
123 within the phylum *Firmicutes*. The type species is *Desulfocucumis palustris*.
124

125 **Description of *Desulfocucumis palustris* sp. nov.**

126 *Desulfocucumis palustris* (pa.lus'tris. L.masc. adj. *palustris*, marshy, swampy).

127 In addition to the properties given in the description of the genus, the following properties are observed. Cells
128 are curved rod to vibroid with 1.0 μm in width and 3.0-4.0 μm and more in length. Motile, with peritrichous
129 flagella. Gram-stain-negative. The colony color is pale brown. Growth occurs at 18-48 °C (optimum, 32-37
130 °C), pH 5.8-8.4 (optimum, 6.2-7.3), and with 0-2.1 % NaCl (optimum, 0%). Sulfate, thiosulfate and elemental
131 sulfur are used as electron acceptors. Sulfite, nitrate, nitrite and Fe (III) are not utilized. The following
132 substrates are utilized as carbon and energy sources in the presence of sulfate: H_2/CO_2 , acetate, propionate,
133 *n*-butyrate, *iso*-butyrate, formate, lactate, fumarate, pyruvate, ethanol and benzoate. Growth fermentatively on
134 lactate, pyruvate, benzoate and fumarate. Major cellular fatty acids are *iso*-C15:0, *iso*-C17:0 and C17:0
135 cyclopropane. No desulfovibridin is detected.

136 The type strain, NAW-5^T (=DSM 102911^T =NBRC 112242^T), was isolated from marsh soil. The genomic
137 DNA G + C content of the type strain is 46.6 mol% (HPLC).
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145 **Conflicts of interest statement**

146 The authors declare that there is no conflict of interest.

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199

200 **Figure legends**

201

202 Fig. 1 Maximum-likelihood tree based on 16S rRNA gene sequence of strain NAW-5^T and the members of
203 the family “Desulfotomaculaceae”. *Peptococcus niger* is used as an outgroup. Number of sequences affiliated
204 with each subclusters is given in parenthesis. Bootstrap values (percentages of 1000 replications) only 50% or
205 more are shown at nodes.

206 Table 1 Characteristics of strain NAW-5^T and relatives. Strains: 1, NAW-5^T; 2, Sulfate-reducing bacterium
 207 strain R-AcetonA170 [16]; 3, *Desulfotomaculum* sp. Ox39 [17]; 4, *Desulfurispora thermophila* RA50E1^T [19].
 208 Positive, +; weakly positive, (+); negative, -; NR, not reported.

209

Characteristic	1	2	3	4
Cell shape	Curved rod or slightly spiral rod	Curved rod	Rod	Rod
Cell length (µm)	3-70	7-8.5	7-8	2-9
Motility	+	+	+	+
DNA G + C content (mol%)	46.6	48.5	NR	53.5
Temperature range (°C)	18-48	NR	NR	40-67
Optimal temperature (°C)	32-37	NR	25-30	59-61
pH range	5.8-8.4	NR	6.5-7.8	6.4-7.9
Optimal pH	6.2-7.3	NR	7.4-7.6	7.0-7.3
NaCl range (%)	0-2.1	NR	NR	0-1
Optimal NaCl (%)	0	NR	NR	0
Electron acceptors:				
Sulfate	+	+	+	+
Sulfite	-	+	-	+
Thiosulfate	+	(+)	-	+
Sulfur	+	-	-	+
Fe(III)	-	-	-	-
Substrates:				
Acetate	+	+	-	-
Propionate	+	+	-	-
Butyrate	+	+	NR	+
iso-Butyrate	+	+	NR	+
Formate	+	-	-	+
Lactate	+	+	-	+
Fumarate	+	-	-	-
Pyruvate	+	-	-	+
Benzoate	+	+	+	-
Glucose	-	NR	-	+