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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-5ᵀ is LC183909.
Abstract

A mesophilic, endospore-forming, sulfate-reducing bacterium, designated as strain NAW-5T was isolated from marsh soil. Cells of strain NAW-5T were Gram-negative staining, curved rods that were motile. Strain NAW-5T grew at 18–48ºC (optimum growth at 32-37 ºC) and pH 5.8-8.4 (optimum growth at pH 6.2–7.3). Electron donors utilized were various organic acids and H₂ which support autotrophic growth. Fermentative growth occurred on carboxylic acids, but not on sugar. Sulfate, thiosulfate and elemental sulfur were used as electron acceptors. The respiratory isoprenoid quinone was MK-7. The genomic DNA G+C content of this strain was 46.6 mol%. Sequence analysis of the 16S rRNA gene showed that strain NAW-5T was affiliated to the family “Desulfotomaculaceae” but the strain shared very low sequence similarity with any representatives of this family (≥89%). Strain NAW-5T belongs to the Desulfotomaculum subcluster Ig which does not include validly named species. On the basis of significant differences in the phylogenetic and phenotypic properties between strain NAW-5T and related species, strain NAW-5T represents novel species of a novel genus for which the name Desulfocucumis palustris gen. nov., sp. nov. is proposed. The type strain is NAW-5T (=DSM 102911T = NBRC 112242T).
The genus *Desulfotomaculum* is a group of obligatory anaerobic, spore-forming sulfate-reducing bacteria [1, 2]. Because of the high phylogenetic divergence, *Desulfotomaculum* species have been classified into seven subclusters, designated as *Desulfotomaculum* subcluster Ia-Ig [3, 4 and 5]. Among these subclusters, only the *Desulfotomaculum* subcluster Ig lacks representative species with a validly published name to date. In this study, a novel sulfate-reducing bacterium belonging to the *Desulfotomaculum* subcluster Ig was isolated and characterized.

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

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

Cellular fatty acids were identified with the Sherlock Microbial Identification System (MIDI) (Sherlock...
Version 6.0; MIDI database MOORE6), and respiratory quinones were analyzed using HPLC [10]. The
presence of desulfoviridin was tested by using fluorescence test described by Postgate [11]. Biomass from
cultures grown at 28ºC with 5 mM acetate and 0.5 g l⁻¹ yeast extract was used for these analyses.

For physiological characterization, each test was performed in duplicate at 28ºC except for tests of growth
temperature. The basal medium containing 10 mM acetate and 0.5 g l⁻¹ yeast extract was used unless
otherwise specified. Temperature range for growth was examined by incubation at 13 different temperatures
(13, 15, 18, 22, 25, 28, 32, 35, 37, 42, 45, 48 and 50 ºC). To determine the pH range for growth, basal
medium was buffered with 20 mM of MES or TAPS instead of NaHCO₃. The pH of the medium was
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,
8.3, 8.4 and 8.7). Range of NaCl concentrations for growth was tested at 6 different concentrations (0, 0.1,
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
of the substrates listed later, and evaluated by monitoring the sulfate reduction. Sulfate reduction was
evaluated based on production of sulfide quantified with the previously described method [12]. Ability for
autotrophic growth with H₂-dependent sulfate reduction was assessed under a gas mixture (H₂/N₂/CO₂,
50:40:10; 2 atm total pressure). Fermentative growth was tested with the media containing no sulfate.
Utilization of electron acceptors was tested using the sulfate-free basal media each containing one of the
substances listed later.

For phylogenetic analysis, genomic DNA of strain NAW-5ᵀ was purified with a Wizard Genomic DNA
Purification Kit (Promega). The 16S rRNA gene fragments were amplified with primers 27f and 1492r [13].
PCR amplification was carried out using Takara Ex Taq DNA polymerase (Takara), and PCR products were
directly sequenced by using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The
obtained nucleotide sequence of the 16S rRNA gene (1442 bp) was aligned with 53 reference sequences from
public databases (GenBank/EMBL/DDBJ) using the program CLUSTAL X version 2.1 [14]. All positions
with gaps were excluded from the calculation, and 1138 positions were included in the final dataset.
Phylogenetic trees were reconstructed with the program MEGA version 7.0.20 [15].

Cells of strain NAW-5T were Gram-negative staining, motile and curved or slightly spiral-shaped rods (1.0
× 3-70 μm) (Fig. S1). The colony color of this strain was pale brown on the anaerobic agar tube containing
basal medium. Peritrichous flagella were observed (Fig S2). Cells of this strain did not make clear swelling
sporangium. Spherical terminal spores were observed. The G+C content of genomic DNA was 46.6 mol%.

Major cellular fatty acids were iso-C15:0 (52.8 %), iso-C17:0 (12.9 %) and cyclopropane-C17:0 (10.0 %).

The other fatty acids detected were C16:0 DMA (4.4 %), C16:0 (2.9 %), C16:0ω7c (2.1 %), iso-C15:0 DMA
(1.5 %), C18:0 (1.5 %), iso-C16:0 (1.3 %), C18:1ω7c (1.3%), aldehyde-C16:0 (0.5 %), C14:0 (0.5 %),
iso-C13:0 (0.3 %), C16:1ω7c DMA (0.3 %), summed features 3 (iso-C15:0 aldehyde; 1.1%) and 10
(C18:1ω7c; 0.5%). Menaquinone-7 (MK-7) was detected as the sole respiratory quinone. Desulfoviridin was
absent.

The growth temperature and pH ranges for growth were 18-48 ºC (optimum, 32-37 ºC), pH 5.8-8.4
(optimum, 6.2-7.3). The NaCl range for growth was 0-2.1 % (optimum, 0%). Under sulfate-reducing
conditions, the strain grew on the following substrates (mM, except where stated): acetate (10), formate (10),
lactate (10), fumarate (5), propionate (5), n-butyrate (5), iso-butyrate (5), pyruvate (5), ethanol (10), methanol
(5), yeast extract (0.5 g l⁻¹) and benzoate (2). Addition of yeast extract greatly enhanced the growth on acetate,
but it was not required for growth on other substrates. Autotrophic growth with H₂ was observed.

Fermentative growth was observed with pyruvate (10), lactate (10) benzoate (5) and fumarate (5). Glucose
(10) could not support growth of the strain under sulfate-reducing conditions or fermentative conditions. In
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
is MK-7. Phylogenetic position based on 16S rRNA gene is located within the family "Desulfotomaculaceae" within the phylum *Firmicutes*. The type species is *Desulfocucumis palustris*.

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

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

In addition to the properties given in the description of the genus, the following properties are observed. Cells are curved rod to vibroid with 1.0 μm in width and 3.0-4.0 μm and more in length. Motile, with peritrichous flagella. Gram-stain-negative. The colony color is pale brown. Growth occurs at 18-48 °C (optimum, 32-37 °C), pH 5.8-8.4 (optimum, 6.2-7.3), and with 0-2.1 % NaCl (optimum, 0%). Sulfate, thiosulfate and elemental sulfur are used as electron acceptors. Sulfite, nitrate, nitrite and Fe (III) are not utilized. The following substrates are utilized as carbon and energy sources in the presence of sulfate: H$_2$/CO$_2$, acetate, propionate, n-butyrte, iso-butyrate, formate, lactate, fumarate, pyruvate, ethanol and benzoate. Growth fermentatively on lactate, pyruvate, benzoate and fumarate. Major cellular fatty acids are iso-C15:0, iso-C17:0 and C17:0 cyclopropane. No desulfoviridin is detected.

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

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Japan Society for the Promotion Science to Watanabe.

**Conflicts of interest statement**

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


Fig. 1 Maximum-likelihood tree based on 16S rRNA gene sequence of strain NAW-5\textsuperscript{T} and the members of the family “Desulfotomaculaceae”. *Peptococcus niger* is used as an outgroup. Number of sequences affiliated with each subclusters is given in parenthesis. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes.
Table 1 Characteristics of strain NAW-5\textsuperscript{T} and relatives. Strains: 1, NAW-5\textsuperscript{T}; 2, Sulfate-reducing bacterium strain R-AcetonA170 [16]; 3, *Desulfotomaculum* sp. Ox39 [17]; 4, *Desulfurispora thermophila* RA50E1\textsuperscript{T} [19].

Positive, +; weakly positive, (+); negative, -; NR, not reported.

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