Desulfosarcina widdelii sp. nov. and *Desulfosarcina alkanivorans* sp. nov., hydrocarbon-degrading sulfate-reducing bacteria isolated from marine sediment and emended description of the genus *Desulfosarcina*

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Abstract

In previous studies, two hydrocarbon-degrading sulfate-reducing bacteria, strains PP31^T and PL12^T were obtained from oil-polluted marine sediments of Shuaiba, Kuwait. They had been reported as organisms capable of anaerobic degradation of *p*-xylene and *n*-alkanes, respectively. The 16S rRNA gene sequence of strain PP31^T showed 98.8 % sequence similarities to that of *Desulfosarcina variabilis* "Montpellier"^{T}. Strains PL12^T had 97.8% of sequence similarity to *Desulfosarcina ovata* strain oXys1^T. They both have been partially characterized, but not been validly published as new species of the genus *Desulfosarcina*. In this study, additional characterizations of these strains were made to describe them as two new species of the genus *Desulfosarcina*. Major cellular fatty acids of strain PP31^T were C15:0 (25.9 %) and anteiso-C15:0 (22.3 %), whereas those of strain PL12^T were C15:0 (21.3 %), C16:0 (17.8 %) and anteiso-15:0 (11.6 %). The phylogenetic tree based on 16S rRNA gene revealed that these isolates should not be classified as any of the known species in the genus *Desulfosarcina*. On the basis of phenotypic and phylogenetic analyses, these two sulfate reducers are proposed to form two novel species of the genus *Desulfosarcina*: *Desulfosarcina widdelii* sp. nov. (PP31^T = JCM 31729^T= DSM 103921^T) and *Desulfosarcina alkanivorans* sp. nov. (PL12^T = JCM 31728^T= DSM 103901^T). In addition, emended description of the genus *Desulfosarcina* is presented in this study.

The genus *Desulfosarcina* is a group of sulfate-reducing bacteria capable of degrading a variety of organic matters, including petroleum hydrocarbons [1]. This genus is currently organized in three species; *D. variabilis*, *D. cetonica* and *D. ovata* [2]. *D. cetonica* DSM 7267^T is able to use toluene as a sole electron donor in the presence of sulfate [3]. *D. ovata* strain oXyS1^T can oxidize toluene and *o*-xylene under sulfate-reducing conditions [1, 4]. As hydrocarbon-degrading sulfate reducers closely related to *Desulfosarcina* species, strains PP31^T and PL12^T were reported in previous studies [5, 6]. Strain PP31^T was reported as the first isolate capable of anaerobic degradation of *p*-xylene [5]. Strain PL12^T was isolated from the same enrichment culture, and demonstrated to degrade *n*-hexane and *n*-decane anaerobically [6]. They both have been partially characterized, but have not been described as *Desulfosarcina* species with validly published names. In this study, additional characterizations about these strains were made to describe them as two new species of the genus *Desulfosarcina*.

Strain PP31^T and PL12^T were obtained from a *p*-xylene-degrading sulfate-reducing enrichment culture established from a petroleum-contaminated sediments of Shuaiba, Kuwait [7]. Basic characterizations of the strains were made in the previous studies [5, 6]. The characteristics reported in the previous studies are summarized in the species description. In the present study, additional characterizations were made to complement the previous studies as described below. The basal medium used for cultivation was a defined bicarbonate-buffered, sulfate-reduced saltwater medium prepared as described by Widdel and Bak [8].

Cell morphology of strain PP31^T was observed by phase-contrast microscopy (Axioplan 2; Zeiss). The Gram-stain test of the strains was carried out by using a Fluka Gram-stain kit as described in the manufacture’s instruction. Analyses of cellular fatty acids 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). Biomass from cultures grown at 28ºC with 5 mM fumarate (strain PP31^T) or 10 mM lactate (strain PL12^T) was used for these analyses.

To determine pH range for growth, the basal media were buffered with 20 mM of MES, TAPS or MOPS instead of $NaHCO_3$. Growth of strain PP31^T was tested with different pH values (5.8, 6.2, 6.5, 7.0, 7.3, 7.5, 7.8, 8.2, 8.3, 8.4, 8.5, 8.7 and 9.0) at 28ºC in the presence of fumarate (5 mM). Utilization of elemental sulfur as an electron acceptor was tested with strain PP31^T using sulfate-free basal medium containing 5 mM propionate at 28ºC. Fermentative growth of strain PP31^T was tested with sulfate-free basal media containing 5mM malate.

For phylogenetic analysis of the 16S rRNA gene, the nucleotide sequences of strains PP31^T and PL12^T were aligned with reference sequences using the program CLUSTAL X version 2.1 [9]. The 16S rRNA gene sequences of the closest relatives were obtained from GenBank/EMBL/DDBJ databases or genomic sequence retrieved from JGI IMG database (http://img.jgi.doe.gov). All positions with gaps were excluded from the calculation. Phylogenetic trees were reconstructed with the program MEGA version 7.0.20 [10].

Cells of strain PP31^T were Gram-negative staining, motile and rod-shaped morphology (0.8 × 6.0 - 27.5 μm). Major cellular fatty acids of strain PP31^T were C15:0 (25.9 %) and anteiso-C15:0 (22.3 %). The other fatty acids detected in strain PP31^T were C17:0 (9.5 %), C15:1 ω6c (6.9 %), C16:0 (6.0 %), C17:1 ω6c (5.4 %), iso-15:0 (4.3 %), C14:0 (2.8 %), C16:1 ω7c (2.3 %), anteiso-C17:0 (2.0 %), C18:0 (1.6 %), summed feature 8 (C17:1 ω8c; 4.0 %) and small amounts (<1%) of C17:0 3OH, C18:1ω9c, C13:0, iso-C15:0 3OH, iso-C16:0, C16:1 ω5c, iso-C14:0, iso-C17:0, C19:0, C14:0 DMA, C16:0 3OH, C18:2 ω6,9c, C18:1 ω5c, anteiso-C19:0, iso-C13:0, summed features 4 (C15:2?), 6 (anteiso-C15:0 3OH), 10 (C18:1 ω7c), 11 (iso-C17:0 3OH). The pH range for growth of strain PP31^T was pH 6.5-8.5 (optimum, 7.3-7.8). In the

presence of 5 mM propionate, strain PP31^T used elemental sulfur as an electron acceptor to support the growth. Cell growth was observed in sulfate-free basal medium containing malate.

Major cellular fatty acids of strain PL12^T were C15:0 (21.3 %), C16:0 (17.8 %) and anteiso-15:0 (11.6 %). The other fatty acids detected in strain PL12^T were C14:0 (9.4 %), C16:1 ω7c (7.6 %), C17:1ω6c (6.0 %), C17:0 (5.9 %), C15:1ω6c (5.9 %), C17:1 ω8c (4.7 %), iso-C15:0 (1.6 %), C18:0 (1.4 %), C16:1 ω5c (1.1 %), summed feature 5 (C15:0 DMA; 1.8 %), 8 (C17:1 ω8c; 3.8 %) and small amounts (<1%) of C17:0 3OH, iso-C15:0 3OH, C16:0 3OH, C13:0, iso-C14:0, iso-C16:0, C18:1 ω5c, C14:0 DMA, C16:1 ω9c, C18:1 ω9c, C19:0, anteiso-C17:0, summed features 4 (C15:2?), 6 (anteiso-C15:0 3OH), 10 (C18:1 ω7c).

Differential characteristics of strains PP31^T, PL12^T and closest relatives are listed in Table 1. As shown in Table 1, strains PP31^T and PL12^T had substantially different properties from other *Desulfosarcina* strains in the hydrocarbon utilization. Strain PP31^T was able to degrade only *p*-xylene, but other hydrocarbons could not support growth of this strain. Strain PL12^T was specialized in *n*-alkane oxidation although other members of the genus *Desulfosarcina* utilized aromatic compounds.

The 16S rRNA gene analysis revealed that the closest cultivated relative of strains PL12^T was *D. ovata* strain oXyS1^T [1, 4]. The sequence similarity between the closest relative and strain PL12^T were 97.8 % (the compared lengths; 1464 bp). Strain PL12^T was closely related to uncultured environmental clone LARHR_86-01F11 [11] with 99.4 % sequence similarity (the compared length; 1464 bp). The closest relative of strain PP31^T was *D. variabilis* strain "Montpellier"^{T} [12], and sequence similarity with the closest relative was 98.8 % (the compared length; 1463 bp). Strains PP31^T and PL12^T were included in the family *Desulfobacteraceae* within the class *Deltaproteobacteria* in the phylogenetic tree (Fig. 1). These strains belonged to the genus *Desulfosarcina* with 100% of bootstrap value support. Strains PP31^T and PL12^T were located in the different positions from other *Desulfosarcina* spp., respectively. This result revealed that these

strains should be distinguished from any of existing species in the genus *Desulfosarcina*. Phylogenetic tree including closely related environmental sequences is shown in supplementary Figure 1.

Based on physiological and phylogenetic characteristics, these two novel sulfate reducers, strains PP31^T and PL12^T obtained from the marine sediments are proposed to form two new species of the genus *Desulfosarcina*: *Desulfosarcina widdelii* sp. nov. (PP31^T = JCM 31729^T= DSM 103921^T) and *Desulfosarcina alkanivorans* sp. nov. (PL12^T = JCM 31728^T= DSM 103901^T),

Emended description of the genus *Desulfosarcina* Widdel 1981.

Cells are packages, oval or rods, occurring singly or in pairs. Spore formation is not observed. Gram-negative. Strictly anaerobic and mesophilic. Sulfate serves as an electron acceptor. Organic electron donors are completely oxidized to CO_2. The G+C content of the DNA is 51-59 mol%. Phylogenetic position based on 16S rRNA gene is located within the family *Desulfobacteraceae* in the class *Deltaproteobacteria*. The type species is *Desulfosarcina variabilis*.

Description of *Desulfosarcina widdelii* sp. nov.

Desulfosarcina widdelii (wid.de′li.i. N.L. gen. masc. n. *widdelii*, of Widdel, named in honor of Friedrich Widdel, a German microbiologist).

Cells are motile and rod-shaped (0.8 × 6.0 - 27.5 μm). Gram-stain-negative. The temperature range for growth is 20-37 ºC, with an optimum at 28 ºC. The pH range for growth is 6.5 – 8.5, with an optimum growth at pH 7.3-7.8. Sulfate, thiosulfate and elemental sulfur are used as electron acceptors with propionate as the

electron donor. Nitrate and sulfite are not used as electron acceptors. In the presence of sulfate, acetate, propionate, succinate, pyruvate, formate, fumarate, *n*-butyrate, benzoate, yeast extract and *p*-xylene are utilized as electron donors and carbon sources. Autotrophic growth on H_2/CO_2 is observed. Lactate, phenol, ethanol, glucose, toluene, benzene, *o*-xylene, *m*-xylene, ethylbenzene, and *n*-hexane are not utilized as electron donors. Growth fermentatively on fumarate and malate, but not lactate. The DNA G+C content of the type strain is 54.8 mol%.

The type strain, PP31^T (=JCM 31729^T =DSM 103921^T), was isolated from the *p*-xylene-degrading sulfate-reducing enrichment culture.

Description of *Desulfosarcina alkanivorans* sp. nov.

Desulfosarcina alkanivorans (al.ka.ni.vo′rans. N.L. n. *alkanum* alkane; L. part. adj. *vorans* devouring; N.L. part. adj. *alkanivorans* devouring alkanes).

Cells are motile and short rod-shaped (0.5 × 1-1.5 μm). Gram-stain-negative. The temperature range for growth is 18-37 ºC, with an optimum at 30-34 ºC. The pH range for growth is 6.5-7.3, with an optimum growth at pH 7.0-7.3. Sulfate, thiosulfate and sulfite are used as electron acceptors with lactate as the electron donor. Elemental sulfur, nitrate, and fumarate are not used as electron acceptors. In the presence of sulfate, lactate, acetate, formate, propionate, butyrate, iso-butyrate, pyruvate, fumarate, yeast extract, *n*-hexane, *n*-decane. Autotrophic growth on H_2/CO_2 is observed. Benzoate, citrate, methanol, ethanol, glucose, phenol, toluene, benzene, ethylbenzene, *o*-, *m*-, *p*-, xylene, cyclohexane, and naphthalene are not utilized as electron acceptors. Cells are incapable of fermentative growth using malate, lactate and fumarate. The DNA G+C content of the type strain is 57 mol%.

The type strain, PL12^T (=JCM 31728^T =DSM 103901^T), was isolated from the *p*-xylene-degrading

sulfate-reducing enrichment culture.

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Conflicts of interest statement

The authors declare that there is no conflict of interest.

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Figure legends

Fig. 1 Maximum-likelihood tree based on 16S rRNA gene sequences of strains PP31^T, PL12^T and representatives of the family *Desulfobacteraceae*. A total of 1463 positions were used in the final dataset. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes.

Table 1 Differential properties of strains PP31^T, PL12^T and *Desulfosarcina* spp.

Strains: 1, *D. widdelii* sp. nov. PP31^T [5]; 2, *D. alkanivorans* sp. nov. PL12^T [6]; 3, *D. ovata* oXyS1^T [1,4]; 4, *D. cetonica* DSM 7267^T [3]; 5, *D. variabilis* "Montpellier"^{T} [12]. Positive, +; weakly positive, (+); negative, -; NR, not reported. * Data obtained from this study.

Characteristics	1	2	3	4	5
Morphology	Rod	Short rod	Rod	Oval	Oval rod, packages
Cell size (µm)	0.8×6-27.5*	0.5 × 1-1.5	0.8–1.0×2.5–4.0	0.8–1.2×1.8–2.7	1–1.5×1.5–2.5
DNA G + C content (mol%)	54.8	57	51	59	51
Optimum temperature (ºC)	28	30-34	32	30	33
Electron donors and carbon sources					
Propionate	+	+	-	NR	NR
Lactate	-	+	+	+	+
Benzoate	+	-	+	+	+
Succinate	+	+	+	-	+
Fumarate	+	+	NR	-	+
n-Hexane	-	+	-	NR	NR
Toluene	-	-	+	+	-
o-Xylene	-	-	+	-	-
p-Xylene	+	-	-	-	-