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***Desulfoluna limicola* sp. nov., a sulfate-reducing bacterium
isolated from sediment of a brackish lake**

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Abstract

A novel sulfate-reducing bacterium, strain ASN36^T, was isolated from sediment of a brackish lake in Japan. Cells of strain ASN36^T were not motile and rod-shaped, with length of 2.4–4.9 µm and width of 0.6–0.9 µm. Growth was observed at 5–35°C with an optimum growth temperature of 28°C. The pH range for growth was 6.6–8.8 with an optimum pH of 7.3. Major fatty acids were C_{16:1} ω7c and C_{16:0}. Under sulfate-reducing conditions, strain ASN36^T utilized lactate, malate, pyruvate, butyrate, ethanol, butanol, glycerol, yeast extract and H₂/CO₂ as growth substrate. Fermentative growth occurred on malate and pyruvate. The novel isolate used sulfate, sulfite and thiosulfate as electron acceptors. The genome of strain ASN36^T is composed of a chromosome with length of 6.3 Mbp and G + C content of 55.1 mol%. Analyses of the 16S rRNA gene indicated that strain ASN36^T is related to *Desulfoluna* species. Overall genome relatedness indices indicated that strain ASN36^T does not belong to any existing species. In contrast to the closest relatives, strain ASN36^T lacks genes for reductive dehalogenase required for organohalide respiration and does not use halogenated aromatics as electron acceptors. On the basis of its genomic and phenotypic properties, strain ASN36^T (= DSM 111985^T = JCM 39257^T) is proposed as the type strain of a new species, *Desulfoluna limicola* sp. nov.

Introduction

The genus *Desulfoluna* was proposed in 2008, with the type species of *D. butyratoxydans* described as a butyrate-oxidizing sulfate reducer (Suzuki et al., 2008). The type strain of the species is *D. butyratoxydans* MSL71^T, isolated from an estuarine sediment by using lactate and sulfate as electron donor and acceptor, respectively. The second species of the genus, *D. spongiiphila* was described with the type strain AA1^T isolated from marine sponge (Ahn et al., 2009). *D. spongiiphila* AA1^T was isolated via enrichment culture with 2-bromophenol as sole electron acceptor, and can dehalogenate various organohalide compounds by respiratory reduction (Ahn et al., 2003; Ahn et al., 2009). Organohalide respiration by *D. butyratoxydans* MSL71^T was demonstrated in a following study, in which another strain of *D. spongiiphila* (strain DBB) was isolated from an intertidal sediment by using 1,4-dibromobenzene as electron acceptor (Peng et al, 2020). All these organohalide-respiring *Desulfoluna* strains are known to have three distinct *rdhA* genes (referred to as *rdhA1*, *rdhA2* and *rdhA3*) in their respective genomes (Peng et al, 2020). These *rdhA* genes, encoding the catalytic subunit of reductive dehalogenase, are individually located in three *rdh* gene clusters scattered in the genomes (Peng et al, 2020). As for two *D. spongiiphila* strains, the involvement of the *rdh* gene clusters in organohalide respiration was investigated by transcriptomic and proteomic

approaches (Peng et al, 2020; Liu et al, 2020), but roles of the multiple gene clusters have not been fully revealed. In addition to these studies on species with validly published names, an enrichment culture-based study suggested organohalide respiration by some *Desulfoluna* species associated with various marine sponges (Horna-Gray et al, 2022).

In this study, a novel sulfate-reducing bacterium was isolated and characterized as a representative of a new species in the genus *Desulfoluna*.

Materials and methods

Enrichment and isolation

The novel isolate, strain ASN36^T was isolated from a sample of sediment collected in a brackish lake, Lake Akkeshi in Japan (43.05° N 144.89° E). The sample was that used in a previous study (Takahashi et al., 2022). To establish the first enrichment, 0.2 g of the sediment was inoculated into a bicarbonate-buffered medium for marine sulfate-reducing bacteria, supplemented with 5 mM butyrate. The medium (hereafter referred to as a basal medium) was prepared as described previously, to contain the following constituents (l⁻¹): 20.0 g NaCl; 4.0 g Na₂SO₄; 3.0 g MgCl₂·6H₂O; 0.2 g KH₂PO₄; 0.25 g NH₄Cl; 0.5 g KCl; 0.15 g CaCl₂·2H₂O; 1 ml trace element solution; 1 ml

selenite-tungstate solution; 1 ml vitamin mixture solution; 1 ml vitamin B₁₂ solution; 1 ml thiamine solution; 30 ml 1 M NaHCO₃ solution; and 1.5 ml 1 M Na₂S solution (Widdel & Bak, 1992). The culture was incubated at 18°C in the dark, after filling the headspace with N₂ /CO₂ (80 : 20, v/v). The grown culture was transferred to the same medium three times (with 3-5 weeks intervals), and then subjected to agar shake dilution as described previously (Widdel & Bak, 1992). A colony formed in the agar-solidified medium was picked up in the liquid basal medium, and the isolation procedures were repeated three times in total to obtain pure culture of strain ASN36^T. The purity of the culture was checked by phase-contrast microscopy and 16S rRNA gene sequencing.

Phenotypic characterization

All cultivation experiments were carried out at 28°C in the basal medium supplemented with 5 mM lactate, unless otherwise specified. Temperature range for growth was examined by culturing strain ASN36^T at 0, 5, 8, 10, 13, 15, 18, 22, 25, 28, 32, 35, 37, 42 and 45°C. Range of salinity for growth was examined by culturing the strain in media with various NaCl concentrations, 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, , 5.0 , 6.0, 7.0, 7.5 and 8.0% (w/v). Effect of pH on growth was tested as described previously (Takahashi et al., 2022), by using media buffered with MES, MOPS, or TAPS. The tested

pH and buffer were as follows: pH 5.8, 6.0, 6.2, 6.4, 6.6 and 6.8 with MES; pH 6.5, 7.0, 7.3, 7.5 and 7.8 with MOPS; 7.8, 8.0, 8.2, 8.4, 8.6, 8.8 and 9.0 with TAPS.

Utilization of electron donors was tested in the basal medium supplemented with one of the following (mM, if not indicated differently): formate (5), acetate (5), propionate (2), lactate (5), glycolate (5), butyrate (5), isobutyrate (5), malate (5), succinate (5), fumarate (5), benzoate (2), pyruvate (5), citrate (5), methanol (5), ethanol (5), butanol (5), glycerol (5), glucose (5) and yeast extract (0.05% w/v). Hydrogen-dependent autotrophic growth was tested under a gas mixture of N₂, H₂ and CO₂ (50:40:10 v/v/v, 200 kPa total pressure). Fermentative growth was tested with a modified version of the basal medium which contained no sulfate. The sulfate-free medium was supplemented with ethanol (5), pyruvate (10), lactate (5), succinate (5), malate (5) or fumarate (5). Acetate in grown cultures was quantified with an Acetate Colorimetric Assay Kit (Sigma-Aldrich). Utilization of electron acceptors was tested in the presence of 5 mM lactate, by replacing sulfate in the basal medium with one of the followings; thiosulfate (10), elemental sulfur (0.5 % w/v), sulfite (1 and 5), tetrathionate (5), poorly crystalline Fe(III) oxide (20), 1,4-dibromobenzene (0.1), 2-bromophenol (0.1), 4-bromophenol (0.1), 2,4-dichlorophenol (0.05) and 2,6-dichlorophenol (0.05).

The cellular fatty acid profile was analyzed for strain ASN36^T grown on 5 mM

lactate. Fatty acids were identified and quantified with the Sherlock Microbial Identification System (MIDI) version 6.0 (database; MOORE6).

Genomic characterization

Whole genome sequencing was performed using the platforms of Illumina NextSeq and Nanopore GridION. The resulting reads from the platforms were subjected to hybrid assembly using Unicycler (Ver 0.4.7). The assembled genome sequence was annotated with DFAST (Tanizawa et al., 2018).

As genome relatedness indices between strain ASN36^T and its closest relatives, values of average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA–DNA hybridization (dDDH) were calculated by using online tools. The values of ANI and AAI were computed with tools provided by Kostas lab (<http://enve-omics.ce.gatech.edu/>). The dDDH was calculated with the formula 2 (Meier-Kolthoff et al., 2013), by using Genome-to-Genome Distance Calculator provided by DSMZ.

The key gene for organohalide respiration, the *rdhA* gene was searched for in the genome of strain ASN36^T, by BLASTp analysis. All nine proteins encoded by three *rdhA* genes (*rdhA1*, *rdhA2* and *rdhA3*) of three *Desulfoluna* strains (*D. butyratoxydans* MSL71^T, *D. spongiiphila* AA1^T and *D. spongiiphila* DBB) were used as queries for

BLASTp search against all proteins encoded in the genome of strain ASN36^T. The *rdhA* genes of three strains were identified in a previous study (Peng et al, 2020).

Phylogenetic analysis based on the 16S rRNA gene

Phylogenetic analysis was conducted using MEGA version 11 (Tamura et al., 2021). The 16S rRNA gene sequence of strain ASN36^T was aligned with reference sequences, obtained from LPSN (accessed on 31 May 2022). With the alignment obtained with the MUSCLE algorithm, the best substitution model was selected by the model selection tool, with default settings. The selected best model with the lowest Bayesian Information Criterion (BIC) score was used to construct phylogenic tree.

Results and Discussion

Physiological and chemotaxonomic characteristics

The characteristics of strain ASN36^T are presented in the species description. Cells of strain ASN36^T were non-motile, rod-shaped, 0.6–0.9 µm in width and 2.0–4.9 µm in length. Strain ASN36^T grew at 5–37°C with optimum growth at 25–30°C. Its pH range for growth was 6.0–8.8 with the optimum pH of 7.3. The NaCl range for growth was 0.5–7.0 %.

Under the sulfate-reducing conditions, growth of strain ASN36^T was supported with lactate, malate, pyruvate, butyrate, ethanol, butanol, glycerol and east extract. In the sulfate-reducing cultures grown on lactate, production of acetate was observed. The molar amount of the generated acetate was nearly equal to that of lactate consumed by sulfate reduction. These results suggest that strain ASN36^T is an incomplete oxidizer, which oxidizes lactate to acetate and CO₂. Chemolithoautotrophic growth was observed in the presence of H₂ gas and CO₂. In the absence of sulfate, fermentative growth was supported by malate and pyruvate. During the fermentative growth, strain ASN36^T generated 2.2 mM acetate from 5 mM pyruvate. Among the alternative electron acceptors tested, only sulfite and thiosulfate were used. None of the tested organohalides supported growth of strain ASN36^T as electron acceptor, in the presence of lactate.

In the cellular fatty acid profile, C_{16:1} *ω*7*c* and C_{16:0} were predominant, accounting for 27.5% and 25.9% of total, respectively. The other major components (>10% of total) were summed feature 10 (C_{18:1} *ω*7*c* and/or unknown 17.834; 11.9%) and C_{18:1} *ω*9*c* (11.5%). The full cellular fatty acid profile is shown in Table S1.

Genomic and phylogenetic properties

The complete genome of strain ASN36^T was reconstructed as a single circular chromosome, with size 6,263,790 bp and G+C content of 55.1%. In the genome, 4971 protein-coding sequences, 12 rRNA genes and 83 tRNA genes were predicted.

The genome harbors four copies of the 16S rRNA gene with an identical sequence. This sequence indicated the highest sequence identities to the 16S rRNA gene sequences of *Desulfoluna* species (Table 1). By constructing phylogenetic tree of the 16S rRNA gene, it was confirmed that strain ASN36^T is closely related to *Desulfoluna* species (Fig. 1). The genome of strain ASN36^T was compared with those of type strains in the genus *Desulfoluna*, by calculating values of ANI, AAI and dDDH (Table 1). All values calculated are lower than threshold for species delineation, suggesting that strain ASN36^T represents a novel species.

In the BLASTp searches with the RdhA sequences from the *Desulfoluna* strains, there was no hit with alignment length greater than 43% of query length. This indicated that none of the genes possessed by strain ASN36^T can be regarded as homologous with the *rdhA1*, *rdhA2* or *rdhA3*. In other words, strain ASN36^T lacks the *rdhA* genes conserved in the other *Desulfoluna* strains.

Conclusion

The analysis of 16S rRNA gene suggested that strain ASN36^T represents a novel species in the genus *Desulfoluna*. The values of ANI, AAI and dDDH between strain ASN36^T and the type strains of *Desulfoluna* species supported the creation of a new species (Table 1). In addition, genomic G + C content of strain ASN36^T is distinct from those of other type strains of *Desulfoluna* species, with differences greater than 2% (Table 1). The differences between strains of same species are 1% or smaller in general (Meier-Kolthoff et al., 2014). Strain ASN36^T also has some phenotypic characteristics, distinct from representatives of existing *Desulfoluna* species (Table 1). On the basis of these results, strain ASN36^T is proposed to be assigned to a new species of the genus *Desulfoluna*, with the name *Desulfoluna limicola* sp. nov., as the first *Desulfoluna* species that does not reduce organohalides.

Description of *Desulfoluna limicola* sp. nov.

Desulfoluna limicola (li.mi'co.la. L. masc. n. *limus*, mud; L. masc. fem. suff. *-cola*, dweller; from L. masc. fem. n. *incola*, dweller; N.L. masc./fem. n. *limicol*, mud-dweller).

Cells are not motile, rod shaped, 2.0–4.9 µm in length and 0.6–0.9 µm in width. Growth

occurs at 5–35°C with an optimum growth at 25–30°C. The pH range for growth is 6.0–8.8, with an optimum pH of 7.3. Predominant fatty acids are C_{16:1} *ω*7c and C_{16:0}. Under sulfate-reducing conditions, grows on lactate, malate, pyruvate, butyrate, ethanol, butanol, glycerol and yeast extract. Formate, acetate, propionate, glycolate, isobutyrate, succinate, fumarate, benzoate, citrate, methanol and glucose are not utilized as growth substrate. Ferments pyruvate and malate. Does not ferment lactate, succinate and ethanol. Grows chemolithoautotrophically on H₂ and CO₂. Uses sulfate, sulfite and thiosulfate as electron acceptor. Elemental sulfur, tetrathionate, poorly crystalline Fe(III) oxide, 1,4-dibromobenzene, 2-bromophenol, 4-bromophenol, 2,4-dichlorophenol and 2,6-dichlorophenol are not used as electron acceptors. G + C content of genomic DNA of the type strain is 55.1 mol%.

The type strain ASN36^T (= DSM 111985^T = JCM 39257^T) was isolated from sediment of a brackish lake in Japan.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and complete genome of strain ASN36^T are LC726356 and AP024488, respectively.

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Statements and Declarations

The authors declare that authors have no relevant financial or non-financial interests to disclose.

Figure legend

Fig. 1. Phylogenetic position of strain ASN36^T, based on the 16S rRNA gene sequences. This maximum likelihood tree was constructed based on Kimura 2-parameter model. A discrete gamma distribution was used to model differences in evolutionary rates among sites (5 categories). The rate variation model allowed for some sites to be invariable (53.53% sites). All positions containing gaps and missing data were eliminated, leaving a total of 1,345 positions in the final dataset. Numbers on nodes represent percentage values of 1,000 bootstrap resampling.

Table 1 Differential properties between strain ASN36^T and type strains of species in the genus *Desulfoluna*. Strains: 1, ASN36^T (this study); 2, *Desulfoluna butyratoxydans* MSL71^T (Suzuki et al., 2008); 3, *Desulfoluna spongiiphila* AA1^T (Ahn et al., 2009). All strains grow on lactate, malate and pyruvate. All strains reduce sulfate, sulfite and thiosulfate. Genome size and G+C content were cited from descriptions in the public database. Organohalide respiration by strain 2 was investigated by Peng et al, 2020.

	1	2	3
Electron donor			
Formate	-	+	+
Succinate	-	-	+
Butyrate	+	+	-
Citrate	-	-	+
Glucose	-	-	+
Electron acceptor			
1,4-Dibromobenzene	-	-	+
2-Bromophenol	-	+	+
4-Bromophenol	-	-	+
Genome size (Mb)	6.26	6.44	6.53
G+C content (mol %)	55.1	57.9	57.2
Relatedness to strain ASN36 ^T			
Identity of the 16S rRNA gene sequence (%)	100	94.8	98.2
ANI (%)	100	81.00	81.26
AAI (%)	100	78.44	79.46
dDDH (%)	100	24.50	24.80

Table S1. Cellular fatty acid profiles of strain ASN36^T, *Desulfoluna butyratoxydans* MSL71^T and *Desulfoluna spongiiphila* AA1^T. Data of strains MSL71^T and AA1^T were taken from Suzuki et al., 2008 and Ahn et al., 2009, respectively. Summed features consist of fatty acids that could not be separated, as follows: Summed feature 2, C_{12:0} 3OH and/or C_{13:0} DMA; Summed feature 3, iso-C_{15:0} aldehyde and/or unknown 13.570; ; Summed feature 5, C_{15:0} DMA and/or C_{14:0} 3OH; Summed feature 10, C_{18:1}ω7c and/or unknown 17.834.

Fatty acid (%)	ASN36 ^T	MSL71 ^T	AA1 ^T
C _{10:0}	2.1	-	-
C _{12:0}	0.5	-	-
iso-C _{14:0}	-	-	10.5±2.8
C _{14:1} ω5c	0.5	-	-
C _{14:0}	8.2	11.4	6.1±0.3
iso-C _{15:0}	-	-	1.9±0.2
anteiso-C _{15:0}	-	-	11.6±0.5
C _{16:1} ω9c	1.2	-	-
C _{16:1} ω7c	27.5	12.9	17.0±2.6
C _{16:1} ω5c	0.5	-	-
C _{16:0}	25.9	27.0	9.8±1.2
iso-C _{16:0}	-	-	2.3±0.1
branched-C _{17:1}	-	1.5	-
C _{18:2} ω6,9c	0.7	-	-
C _{18:1} ω9c	11.5	7.5	-
C _{18:0}	1.1	2.1	3.7±1.8
C _{20:0}	0.1	-	-
Summed feature 2	0.6	-	-
Summed feature 3	0.3	-	-
Summed feature 5	7.3	8.9 ^a	14.9±2.7 ^a
Summed feature 10	11.9	17.8 ^b	23.8±0.9 ^b

a: Reported as C_{14:0} 3OH. *b*: Reported as C_{18:1}ω7c.

