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Oleomonas sagaranensis gen. nov., sp. nov., represents a novel genus in the α-*Proteobacteria*

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Abstract

A Gram-negative bacterium was previously isolated from an oil field in Shizuoka, Japan, and designated strain HD-1. Here we have performed detailed characterization of the strain, and have found that it represents a novel genus. The 16S rRNA sequence of strain HD-1 displayed highest similarity to various uncultured species (86.7∼99.7%), along with 86.2∼88.2% similarity to sequences from *Azospirillum*, *Methylobacterium*, *Rhizobium*, and *Hyphomicrobium*, all members of the α-*Proteobacteria*. Phylogenetic analysis revealed that HD-1 represented a deep-branched lineage among the α-*Proteobacteria*. DNA–DNA hybridization analysis with *Azospirillum lipoferum* and *Hyphomicrobium vulgare* revealed low levels of similarity among the strains. We further examined the biochemical properties of the strain under aerobic conditions. Among carbon sources, ethanol, *n*-propanol, *n*-butanol, and *n*-tetradecanol were the most preferred, while acetate, propionate, and pyruvate also supported high levels of growth. The strain could also grow on aromatic compounds such as toluene, benzene and phenol, and aliphatic hydrocarbons such as *n*-octane and *n*-tetradecane. In contrast, glycerol and various sugars, including glucose, fructose, maltose, and lactose, failed to support growth of HD-1. Under an anaerobic gas phase with butanol as the carbon source, little increase in cell weight was observed with the addition of several possible electron acceptors. As strain HD-1 represents a novel genus in the α-*Proteobacteria*, we designated the strain as *Oleomonas sagaranensis* gen. nov., sp. nov., strain HD-1.

Keywords: α-*Proteobacteria*; *Oleomonas sagaranensis*

1. Introduction

The isolation of a Gram-negative bacterium from an oil field in Shizuoka, Japan, has previously been reported [1]. The strain was designated as HD-1, and various biochemical properties related with hydrocarbon metabolism under aerobic and anaerobic conditions have been examined [1–3]. HD-1 was found to be a mesophilic bacterium growing well at temperatures between 28 and 37°C. HD-1 cells were rod-shaped, with a size of approximately 0.5 μm× 1.2–1.5 μm. The cell surface was remarkably thick, and the lipophilic fraction of HD-1 cells was extremely high (16–23%) compared to typical bacterial strains such as *Escherichia coli* (7%) and *Bacillus subtilis* (4%). Some enzymes from strain HD-1 have also been examined, and biochemical characterizations of an aldehyde dehydrogenase [4] and esterase [5] from this strain have been reported. Both enzymes were found to prefer hydrophobic substrates [4,5]. As we had not focused on the taxonomy of the strain in previous reports, here we aimed to identify the strain in terms of its phylogenetic position and clarify its biochemical and/or physiological characteristics. We have examined in detail the carbon and energy sources that the strain prefers, along with the utilization of electron acceptors. Moreover, based on the 16S rRNA sequence and results of DNA–DNA hybridization, we

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have found that strain HD-1 represents a deep-branched lineage among the α-*Proteobacteria*. From these results, we designate the strain as *Oleomonas sagaranensis* gen. nov., sp. nov.

2. Materials and methods

2.1. Strains

Strain HD-1 was previously isolated from an oil field in Sagara, Shizuoka, Japan [1]. *E. coli* strain DH5α was used for gene cloning and manipulation. *E. coli* strain XL1-Blue MRA(P2) (Stratagene, La Jolla, CA, USA) was used as a host cell for the λEMBL3 genomic DNA library. *Azospirillum lipoferum* (JCM1227, ATCC29708) and *Hyphomicrobium vulgare* (JCM6889, ATCC33404) were purchased from the Japan Collection of Microorganisms (JCM, Wako, Japan).

2.2. DNA manipulations

Routine DNA manipulations were performed by standard methods [6]. A λEMBL3 genomic DNA library of HD-1 was prepared by ligating genomic DNA partially digested with *Sau*3AI into *Bam*HI-digested arms of λEMBL3 (Stratagene). For isolation of plasmid and phage DNA, plasmid mini-, midi- and lambda kits (Qiagen, Hilden, Germany) were used. Genomic DNA was isolated with Genomic-tip 100/G (Qiagen). Restriction enzymes, DNA polymerase and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan).

2.3. Gene cloning and DNA sequencing

A partial fragment of the 16S rRNA gene of strain HD-1 was amplified by polymerase chain reaction (PCR) in a GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Universal small-subunit rRNA primers (5′-AAACT(C/T)AAA(G/T)GAATTGACGG-3′ and 5′-ACGGGCGGTGTGT(A/G)C-3′) corresponding to nucleotide positions 907–926 and 1406–1392 of the *E. coli* 16S rRNA gene, respectively, were used [7]. The PCR product (0.5 kbp) was labeled with the DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany) and used as a probe to clone the full-length 16S rRNA gene. DNA sequencing on both strands of DNA was performed using the ABI Prism kit and Model 310 capillary DNA sequencer (Perkin-Elmer Applied Biosystems). The sequence data was analyzed using Genetyx-Win Version 4 software package (Software Development, Tokyo, Japan). The multiple alignment of DNA sequences was performed using the program Align contained within the ClustalW program [8] provided by DNA Data Bank of Japan (DDBJ). A phylogenetic tree was constructed by the neighbor-joining method [9] by comparing the sequences of a 1317-bp region of various 16S rRNA genes. Bootstrap resampling was performed with the Bstrap program 1000 times. The tree topology was confirmed by the maximum-likelihood method [10] with the fastDNAmL program [11].

2.4. DNA-DNA hybridization

DNA–DNA hybridization was carried out at Higeta Shoyu (Chiba, Japan) using photobiotin (Vector Laboratories, Burlingame, CA, USA) labeled probes in microplate wells as described elsewhere [12,13]. Genomic DNA from strain HD-1, *A. lipoferum*, and *H. vulgare* were compared. Templates and biotinylated probe DNAs were used at levels of 1 μg and 20 ng per well, respectively. Hybridization was performed at 37°C for 12 h. The DNA reassociation ratios were determined by using streptoavidin-peroxidase (Zymed Laboratory, San Francisco, CA, USA) and 3,3′,5,5′-tetramethylbenzidine (Dojin Kagaku, Kumamoto, Japan) and were estimated with a microplate reader at 620 nm.

2.5. Nucleotide sequence accession number

The 16S rRNA gene sequence of HD-1 is available under the accession no. D45202 in the GenBank/EMBL/DDBJ databases.

2.6. Substrate utilization of strain HD-1

In order to determine the carbon sources that are utilized by HD-1, cells were cultivated in a modified BS medium [14] containing the following (per liter of deionized water): KH_2PO_4, 1.4 g; NH_4Cl, 0.25 g; $NaHCO_3$, 0.22 g; $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, 0.2 mg; $Na_2WO_4{\cdot}2H_2O$, 0.1 mg; Na_2SeO_4, 0.1 mg; trace mineral solution [15], 10 ml. Trace mineral solution contains (per liter of deionized water): nitrilotriacetic acid, 1.5 g; $MgSO_4{\cdot}7H_2O$, 3.0 g; $MnSO_4{\cdot}2H_2O$, 0.5 g; NaCl, 1.0 g; $FeSO_4{\cdot}7H_2O$, 0.1 g; $CoCl_2$, 0.1 g; $CaCl_2{\cdot}2H_2O$, 0.1 g; $ZnSO_4$, 0.1 g; $CuSO_4{\cdot}5H_2O$, 0.01 g; $AlK(SO_4)_2$, 0.01 g; H_3BO_3, 0.01 g; $Na_2MoO_4{\cdot}2H_2O$, 0.01 g. When added, the final concentrations of the following substrates were 0.1% (w/v): yeast extract, tryptone, casamino acids, nitrogen base w/o amino acids, nutrient broth, NZ amine, glucose, maltose, glucitol, lactose, arabitol, sucrose, rhamnose, mannitol, inositol, sorbitol, fructose, xylose, xylitol, soluble starch, formate, acetate, propionate, *n*-butyrate, citrate, pyruvate, succinate, fumarate, maleate, malate, lactate, glutamate, *n*-tetradecane, *n*-octane, glycerol, methanol, *n*-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, *n*-decanol, *n*-dodecanol, *n*-tetradecanol, *n*-hexadecanol, *n*-octadecanol, formaldehyde, formamide, toluene, benzene, xylene, and aniline. The concentration of ethanol was 0.05% and that for phenol was 0.01%. As benzene, toluene and cyclohexane are highly

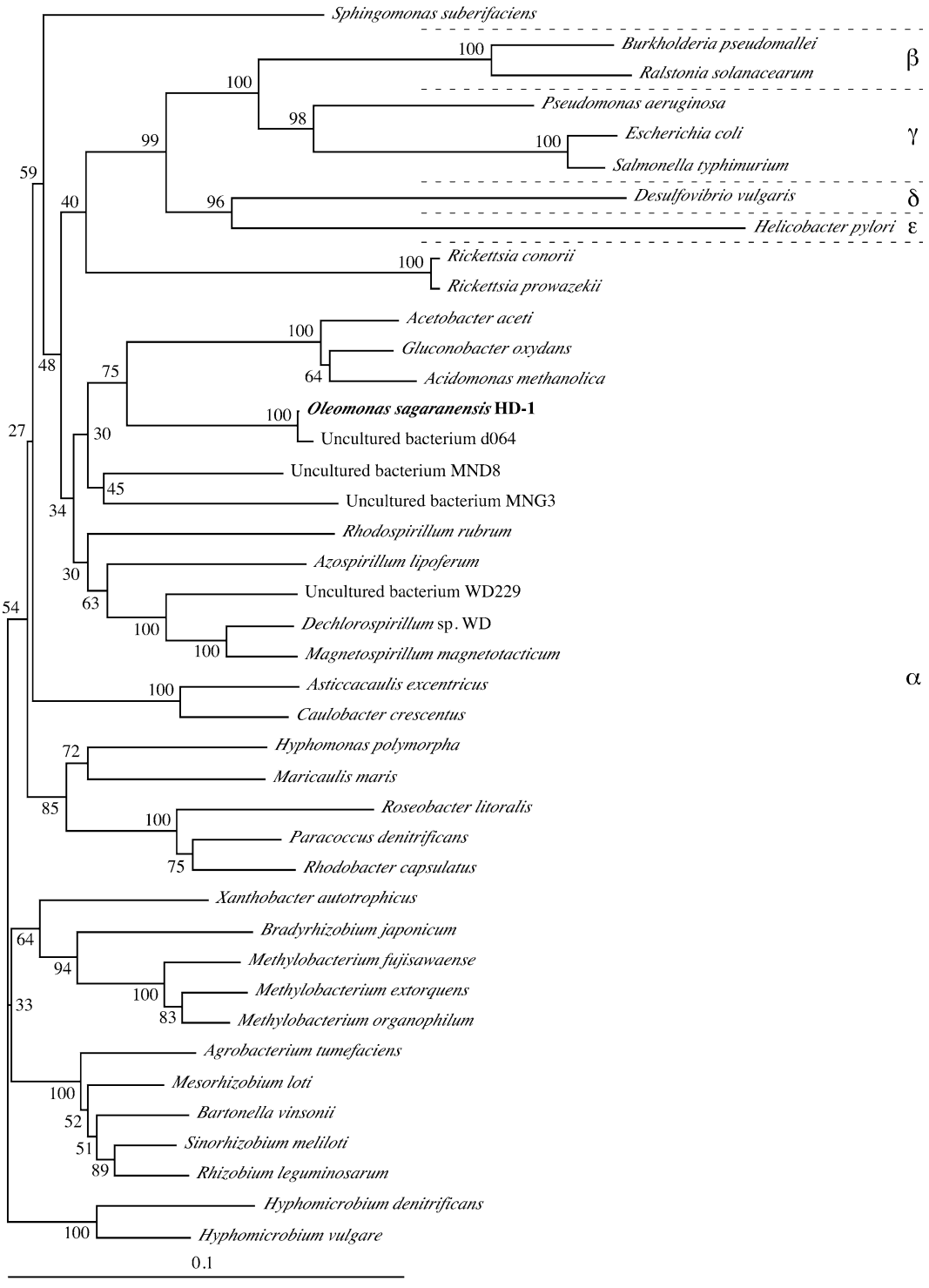

Fig. 1 (Caption overleaf).

volatile, these substrates were repeatedly added (0.1% (w/v)) to the culture every 12 h.

2.7. Electron acceptors under anaerobic conditions

When we investigated the electron acceptors, HD-1 was cultivated anaerobically in the modified BS medium, supplemented with 10 mM butanol. Resazurin (1 mg l^{-1}) was used as a redox indicator. Oxygen in the medium was reduced by adding 0.03% $Na_2S{\cdot}9H_2O$ (w/v). The following compounds, which were possible electron acceptors, were added to the medium at a final concentration of 10 mM; sodium thiosulfate pentahydrate, sodium sulfate, sodium sulfite, sodium nitrate, sodium nitrite, ferric citrate, and

Fig. 1. Phylogenetic tree based on the 16S rRNA sequence information. Calculations were performed by the ClustalW program provided by DNA Data Bank of Japan (DDBJ). Segments corresponding to an evolutionary distance of 0.1 are shown. Each name at the termini represents the species from which the 16S rRNA originated. The accession numbers for each sequence are as follows: *Acetobacter aceti*, D30768; *Acidomonas methanolica*, D30770; *Agrobacterium tumefaciens*, NC_003304; *Asticcacaulis excentricus*, AB016610; *Azospirillum lipoferum*, Z29619; *Bartonella vinsonii*, L01259; *Bradyrhizobium japonicum*, U69638; *Burkholderia pseudomallei*, NC_002930; *Caulobacter crescentus*, NC_002696; *Dechlorospirillum* sp. WD, AF170352; *Desulfovibrio vulgaris*, NC_002937; *Escherichia coli*, NC_000913; *Gluconobacter oxydans*, X73820; *Helicobacter pylori*, NC_000915; *Hyphomicrobium denitrificans*, Y14308; *Hyphomicrobium vulgare*, Y14302; *Hyphomonas polymorpha*, AJ227813; *Magnetospirillum magnetotacticum*, Y10110; *Maricaulis maris*, AJ227802; *Mesorhizobium loti*, NC_002678; *Methylobacterium extorquens*, D32224; *Methylobacterium fujisawaense*, AJ250801; *Methylobacterium organophilum*, D32226; *Paracoccus denitrificans*, Y16927; *Pseudomonas aeruginosa*, NC_002516; *Ralstonia solanacearum*, NC_003295; *Rhizobium leguminosarum*, U29386; *Rhodobacter capsulatus*, D16428; *Rhodospirillum rubrum*, D30778; *Rickettsia conorii*, NC_003103; *Rickettsia prowazekii*, NC_000963; *Roseobacter litoralis*, X78312; *Salmonella typhimurium*, NC_003197; *Sinorhizobium meliloti*, NC_003047; *Sphingomonas suberifaciens*, D13737; *Oleomonas sagaranensis* strain HD-1, D45202; Uncultured bacterium d064, AF422655; uncultured bacterium MND8, AF292999; uncultured bacterium MNG3, AF293000; uncultured bacterium W229, AJ292593; *Xanthobacter autotrophicus*, X94201. Abbreviations are as follows: α, α-*Proteobacteria*; β, β-*Proteobacteria*; γ, γ-*Proteobacteria*; δ, δ-*Proteobacteria*; ε, ε-*Proteobacteria*.
←

sodium chlorate. In the case of sodium chlorate, 10 mM acetate was also used as an electron donor instead of butanol.

2.8. Antibiotics sensitivity

In order to determine the sensitivity against antibiotics, HD-1 was cultivated in LB medium. Chloramphenicol, penicillin G, rifampicin, streptomycin, ampicillin, gentamicin, kanamycin, neomycin and carbenicillin were tested at final concentrations of 25, 50 and 100 μg ml^{-1} in three independent experiments. Tetracycline was tested at a concentration of 12.5 μg ml^{-1}. Cultures were inoculated in duplicates for all experiments and cells were grown for four days at 35°C aerobically.

3. Results

3.1. Phylogenetic analysis of strain HD-1

The entire 16S rRNA gene from strain HD-1 was cloned and the nucleotide sequence was determined on both strands. A database search for sequences similar to the HD-1 sequence was performed. The most similar sequences were found to be environmental sequences of various uncultured bacteria (86.7∼99.7%) [16,17]. Among previously identified and characterized bacteria, sequences from *Azospirillum*, *Methylobacterium*, *Rhizobium*, and *Hyphomicrobium*, all members of the α-*Proteobacteria*, displayed high similarity (86.2∼88.2%). A region of 1317 bp (position 41 to 1358 in the HD-1 sequence) in length was aligned with other available 16S rRNA sequences of various bacteria, particularly α-*Proteobacteria*. A phylogenetic tree was constructed by the neighbor-joining method (Fig. 1) and maximum-likelihood method (not shown). The results clearly displayed that strain HD-1 represented a deep-branched lineage among α-*Proteobacteria*, suggesting that it was a member of a novel genus.

3.2. DNA–DNA hybridization

We performed DNA–DNA hybridization with two members of α-*Proteobacteria* that displayed high similarity in 16S rRNA sequences: *Azospirillum lipoferum* and *Hyphomicrobium vulgare*. The genomic DNA of strain HD-1 exhibited very low hybridization signals with the isolated genomic DNA of *A. lipoferum* (9%) and *H. vulgare* (33%) (Table 1). Genomic DNA from *A. lipoferum* and *H. vulgare* displayed 38% homology with each other.

3.3. Antibiotic sensitivity

We investigated the sensitivity of strain HD-1 against various antibiotics; chloramphenicol, penicillin G, rifampicin, streptomycin, ampicillin, gentamicin, kanamycin, neomycin, carbenicillin, and tetracycline. Strain HD-1 was sensitive to all antibiotics examined at concentrations of 25 μg ml^{-1}.

3.4. Utilization of carbon sources

In order to determine the carbon sources that strain HD-1 could utilize, we added various substrates to BS medium and examined cell growth (Table 2). Cell growth was monitored by cell weight, as HD-1 cells have a tendency to aggregate. HD-1 was able to grow on proteinaceous complex substrates such as yeast extract, tryptone,

Table 1
DNA–DNA hybridization (% homology) of strain HD-1 and species of genus *Azospirillum* and *Hyphomicrobium*

Source of microplate-bound DNA	Homology (%) with the photobiotin-labeled DNA of:		
	Strain HD-1	*Azospirillum lipoferum* (JCM 1227)	*Hyphomicrobium vulgare* (JCM 6889)
Strain HD-1	100	9	33
Azospirillum lipoferum	–	100	38

nutrient broth, peptone, or casamino acids but not on yeast nitrogen base w/o amino acids. Among the proteinaceous complex substrates, yeast extract proved to be the best substrate for HD-1. High levels of cell growth were observed for various alcohols, ethanol, *n*-propanol, *n*-butanol and *n*-tetradecanol. Other carbon sources that led to notably high growth levels were pyruvate, acetate, propionate, and phenol. Strain HD-1 was also able to utilize other aromatic compounds such as toluene and benzene, as well as aliphatic (*n*-octane or *n*-tetradecane) hydrocarbons. Interestingly, no growth was observed on various sugar compounds. These included glucose, lactose, arabitol, sucrose, glucitol, rhamnose, mannitol, inositol, sorbitol, fructose, xylose, maltose, and xylitol. As several members of α-*Proteobacteria* can utilize C1 compounds [18], we also analyzed growth on methanol, formaldehyde, formate, and formamide. No growth was observed with any of these C1 compounds.

3.5. Electron acceptors

We further investigated whether strain HD-1 could grow under anaerobic conditions. We performed anaerobic culture with BS medium with 0.1% butanol, supplemented with various candidate electron acceptors. The anaerobic gas phase was composed of $N_2:CO_2:H_2 = 90:5:5$, and 0.03% (w/v) $Na_2S{\cdot}9H_2O$ was added to reduce trace amounts of oxygen. The electron acceptors added in the experiments are shown in Table 3. Without the addition of electron acceptors, no growth was observed. A slight increase in cell weight was observed when nitrite, sulfite and thiosulfate were added, but growth levels were extremely low compared to aerobic conditions. As *Dechlorospirillum*, one of the phylogenetic neighbors, can reduce chlorate coupled with the oxidation of acetate [19], we also investigated the growth of strain HD-1 with addition of acetate and chlorate. However, no growth was observed.

Table 2
Comparison of growth of strain HD-1 on various substrates

Carbon source	Wet weight (mg/5 ml culture)	Carbon source	Wet weight (mg/5 ml culture)
Control (no substrate added)		Organic acids	
	1.2	acetate	3.8
		propionate	2.5
C1 compounds		*n*-butyrate	1.0
methanol[a]	2.0	citrate[a]	1.1
formaldehyde	1.6	succinate	2.0
formate	1.5	pyruvate	6.5
formamide	1.2	lactate	2.6
		fumarate	1.1
Aliphatic hydrocarbons		maleate	0.7
n-octane	2.8	malate[a]	1.6
n-tetradecane[a]	2.9	glutamate	2.1
Aromatic hydrocarbons		Sugars	
benzene[a]	2.9	glycerol[a]	1.4
toluene[a]	2.8	glucose[a]	1.5
phenol	4.5	glucitol	1.4
xylene	1.1	lactose	1.3
aniline	1.1	arabitol	1.2
		sucrose[a]	1.2
Alcohols		rhamnose	1.1
ethanol[a]	6.3	mannitol	1.4
n-propanol[a]	12.4	inositol	1.3
n-butanol[a]	7.0	sorbitol	1.3
n-hexanol[a]	2.5	fructose	1.4
n-octanol	1.7	xylose	1.5
n-decanol	2.2	xylitol	1.2
n-dodecanol	5.5	maltose[a]	1.2
n-tetradecanol	15.0	soluble starch	1.5
n-hexadecanol	3.3		
n-octadecanol	1.5	Proteinaceous complex substrates	
		yeast extract	5.0
		tryptone	3.0
		casamino acids	2.3
		NZ amine	2.6
		yeast nitrogen base w/o amino acids	1.2
		nutrient broth	2.8

[a]Substrates that have been examined in the reference [1].

Table 3
Comparison of growth of strain HD-1 on various possible electron acceptors

Electron acceptor	Electron donor (carbon source)	Wet weight (mg/5 ml culture)
-	butanol	0.063
nitrite	butanol	0.131
nitrate	butanol	0.056
sulfite	butanol	0.125
thiosulfate	butanol	0.100
sulfate	butanol	0.031
chlorate	butanol	0.082
chlorate	acetate	0.069

4. Discussion

In this study we have performed further characterization of a previously isolated strain HD-1. The 16S rRNA sequence, along with the results of DNA–DNA hybridization clearly indicate that the strain represents a novel genus in the α-*Proteobacteria* subdivision. We therefore designate the strain as *Oleomonas sagaranensis* HD-1.

Strain HD-1 has previously been reported to be capable of growth under anaerobic conditions [1]. However, in this study, we could not detect significant growth of the strain with several possible electron acceptors under anaerobic conditions. Maximum growth was less than 2% of that observed in the same media under aerobic conditions. Considering that sulfide was not added to the medium in the previous report, strain HD-1 may grow only under microaerobic conditions.

The α-subdivision of *Proteobacteria* is a very diverse group of microorganisms. For example, this subdivision involves not only chemoorganotrophs that utilize various carbon sources, including toxic compounds such as aromatics (*Sphingomonas*) [20] and chlorides (*Sphingomonas*, *Methylobacterium*) [21,22], but also phototrophs (*Rhodobacter*) [23] and chemolithotrophs (*Paracoccus*) [24]. Another member is the unique *Magnetospirillum*, which forms magnetically charged intracellular iron crystals called magnetosomes [25]. The subdivision also includes bacteria that are associated with various plants and animals. Some of them, such as the symbiotic nitrogen-fixing bacteria belonging to the genus of *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, make vital contributions to nitrogen uptake in the hosts [26], while some parasites such as *Rickettsia prowazekii*, the cause of epidemic typhus [27], are notorious pathogens. The morphology of these microbes is also very diverse, including coccoidal, rod-shaped, spiral-shaped, and stalked cells. The only common characteristic that unites them besides their phylogeny might be that they are all Gram-negative.

Several members of the α-*Proteobacteria* have been shown to utilize C1 compounds. *Methylobacterium* species aerobically assimilate methane, methanol, and formaldehyde. *Hyphomicrobium* species utilize methanol, formaldehyde, formate, and methylamine. In contrast, strain HD-1 did not exhibit growth on any of the examined C1 compounds.

Another distinct feature of strain HD-1 was the inability of the strain to utilize sugar compounds as a carbon source. Several species belonging to the genus of *Azospirillum*, *Rhizobium*, and *Mesorhizobium* utilize sugar compounds such as galactose, arabinose, and xylose. Glucose is assimilated by *Methylobacterium* and *Rhizobium* species. These neighbors also grow well on organic acids such as lactate, citrate, succinate, pyruvate, and malate. However, strain HD-1 displayed high levels of growth only in the case of pyruvate. Instead, we found that strain HD-1 preferred various hydrocarbons. The aromatic benzene, toluene and phenol, along with aliphatic hydrocarbons, supported growth, in good accordance to the natural environment where the strain was originally isolated. The ability to degrade and/or utilize hydrocarbon compounds has not been reported as of yet in the cases of *Azospirillum*, *Hyphomicrobium*, *Rhizobium*, *Methylobacterium*, and *Mesorhizobium*. The distant phylogenetic position of the strain, along with its characteristic physiological properties, makes strain HD-1 an attractive target in the field of research on α-*Proteobacteria*.

5. Description of *Oleomonas sagaranensis* gen. nov. sp. nov.

Oleomonas sagaranensis (o.le.o.mo.nas. sa.ga.ra.nen'sis. L. neut. n. *oleum* oil; L. fem. n. *monas* monad; M.L. fem. n. *oleomonas*; M.L. fem. adj. *sagaranensis* pertaining to Sagara, where the oil contaminated soil used to isolate the strain was sampled).

Rod-shaped cells are usually 1.2–1.5 μm long and 0.5 μm wide. Gram-negative. Motile. Cells grown on 1.0% tryptone and 0.5% yeast extract tend to aggregate. Growth occurs between 25 and 40°C (optimum: 37°C), at pH 5.5–7.5 (optimum: at 6.2–6.5), and at 0–2.0% NaCl (optimum: 0.5%). Colonies on nutrient-rich agar plate medium are yellowish-white, rough, and raised, usually with a diameter of 2 mm. Colonies on nutrient-poor agar plate medium are yellowish-white or white, and flat, usually with a diameter of 3–4 mm. Nitrate reduction negative, indole production negative, and arginine dihydrolase negative. Hydrolysis of esculin, gelatin and *p*-nitro-phenyl-β-D-galactopyranoside

was not detected. Catalase and oxidase positive. Utilizes diverse hydrocarbons and alcohols. Ethanol, *n*-propanol, *n*-butanol, and *n*-tetradecanol lead to particularly high levels of growth. Pyruvate is another effective carbon substrate, although only slight or no growth occurs with most sugar compounds and organic acids. The G+C content of genomic DNA is about 66 mol%. The 16S rRNA sequence displays only 88.0% similarity to *Azospirillum lipoferum*, 87.9% to *Methylobacterium organophilum*, 87.9% to *Magnetospirillum magnetotacticum*, and 87.2% to *Mesorhizobium loti*.

Type strain: *Oleomonas sagaranensis* HD-1 (isolated from Sagara, Shizuoka, Japan), FERM P-18643, International Patent Organism Depositary National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaragi 305-8566, Japan.

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