Desulfoglaeba alkanexedens gen. nov., sp. nov., an n-alkane-degrading, sulfate-reducing bacterium

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Two novel sulfate-reducing bacteria, strains ALDC^T and Lake, which were able to oxidize n-alkanes, were isolated from a naval oily wastewater-storage facility (VA, USA) and from oilfield production water (OK, USA), respectively. The type strain (ALDC^T) had a narrow substrate specificity and could grow only with n-alkanes (from C₆ to C₁₂), pyruvate, butyrate, hexanoic acid and 4-methyloctanoic acid. Cells of strain ALDC^T stained Gram-negative and were slightly curved, short rods with oval ends (2.5–3.0 × 1.0–1.4 µm), often occurring in pairs. Cells tended to form aggregates or large clusters and were non-motile and did not form endospores. Optimum growth occurred between 31 and 37 °C and at pH 6.5–7.2. NaCl was not required for growth, but salt concentrations up to 55 g l⁻¹ could be tolerated. The DNA G + C content was 53.6 mol%. Phylogenetic analysis of the 16S rRNA genes revealed that strains ALDC^T and Lake were closely related, but not identical (99.9% similarity). The two strains were not closely related to other known alkane-degrading, sulfate-reducing bacteria or to other genera of the *Deltaproteobacteria*. Therefore, it is proposed that strain ALDC^T (=JCM 13588^T=ATCC BAA-1302^T) represents the type strain of a novel species and genus, with the name *Desulfoglaeba alkanexedens* gen. nov., sp. nov.

INTRODUCTION

Anaerobic biodegradation of n-alkanes is a well-documented process that has been demonstrated under nitrate-reducing, sulfate-reducing and methanogenic conditions. n-Alkanes are metabolized under anaerobic conditions by at least two mechanisms that involve the addition of carbon to the parent molecule. One activation mechanism involves a direct carboxylation reaction from bicarbonate (So *et al.*, 2003), whereas the other involves addition of the alkane to the double bond of fumarate with the formation of alkylsuccinic acid derivatives (Kropp *et al.*, 2000; Rabus *et al.*, 2001).

Several sulfate-reducing and denitrifying bacterial strains capable of the complete conversion of n-alkanes to CO₂ have been isolated (Aeckersberg *et al.*, 1991, 1998; Rueter *et al.*, 1994; So & Young, 1999; Ehrenreich *et al.*, 2000; Cravo-Laureau *et al.*, 2004). A few of the novel strains have been fully characterized. All known alkane-degrading, nitrate-reducing bacteria (strains HxN1, OcN1 and HdN1) belong to the *Betaproteobacteria* *Azoarcus*/*Thauera* group and *Deltaproteobacteria* (Ehrenreich *et al.*, 2000). All of the

sulfate-reducing, alkane-degrading isolates are short oval-shaped rods belonging to the *Deltaproteobacteria*. Morphologically and physiologically, the sulfate-reducing isolates (with the exception of a thermophilic strain, TD3) are generally similar. However, phylogenetic comparison reveals substantive differences amongst the organisms. Comparison of 16S rRNA gene sequences reveals that several sulfate-reducing isolates cluster close to strain AK-01, including the mesophilic strains Pnd3, Hxd3 and the recently isolated *Desulfatibacillum aliphaticivorans* strain CV2803^T. It has been suggested that these strains represent members of the genus *Desulfatibacillum* (Cravo-Laureau *et al.*, 2004).

In this paper, we describe two novel alkane-degrading, sulfate-reducing bacteria, strain ALDC^T, isolated from sludge collected from a naval oily wastewater-storage facility and strain Lake, isolated from production water from an oilfield in Oklahoma (USA). The two strains are phylogenetically close to each other but not to members of the genus *Desulfatibacillum*. We propose that strain ALDC^T represents the type strain of a novel species and genus.

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ALDC^T and Lake are DQ303457 and DQ303458, respectively.

METHODS

Source of organisms. Strain ALDC^T was isolated from sludge collected from a naval, oily wastewater-storage facility at the US Navy

Craney Island Fuel Depot in Portsmouth, VA, USA. Strain Lake was isolated from produced water collected from the Bebee-Konawa oilfield in Oklahoma.

Enrichment and isolation. Enrichment cultures yielding strain ALDC^T were obtained using a basal sulfate-containing, brackish water mineral medium (Widdel & Bak, 1992), as described previously (Kropp *et al.*, 2000). Anoxic incubation of produced water collected from an oil-water separation tank in the Bebee-Konawa oilfield actively reduced sulfate in the presence of crude oil. Cells from this water served as an inoculum for enrichments that ultimately yielded strain Lake. The Lake enrichments were obtained using the same basal sulfate-containing mineral medium, but for freshwater conditions (Widdel & Bak, 1992). After sterilization, the two media were supplemented with (per litre): 3.0 g NaHCO₃ (from 10% stock solution), 10 ml RST vitamin solution without mercaptoethansulfonic acid (Tanner, 1997), 0.05 g Na₂S.9H₂O and 0.05 g cysteine-HCl. The final pH was 7.2. Aliquots (25 ml) of the media were distributed in 160 ml serum bottles using anaerobic technique (Balch *et al.*, 1979; Hungate, 1969) and were supplemented with 0.5 ml of an alkane mixture, comprising hexane/decane/dodecane/hexadecane (1:1:1:1, by vol.), which was supplied neat from anoxic (flushed with N₂) and autoclaved stock solutions. The incubation bottles were sealed with Teflon-lined stoppers, secured with aluminium crimp seals and incubated inverted under a N₂/CO₂ (80:20) gas phase at 31 °C. Growth of the cultures was inferred by following the reduction of sulfate. Significant losses of electron acceptor (sulfate) relative to the substrate-unamended and sterile controls indicated the development of the desired activity. Positive incubations were used for further enrichment. After repeated transfers, stable cultures that contained cells of the same predominant morphology were obtained. These cultures were assayed for alkane loss as described previously (Kropp *et al.*, 2000) and the stoichiometry of alkane consumption and sulfate reduction was determined. Cultures with the expected stoichiometry were selected for further purification.

Pure cultures were obtained by repeated serial dilution in basal seawater medium (Widdel & Bak, 1992), supplemented with RST trace metal solution (10 ml l⁻¹) and modified RST vitamin solution (10 ml l⁻¹) (Tanner, 1997) supplemented with 5.0 mg 6,8-thioctic acid l⁻¹ instead of mercaptoethansulfonic acid. The final pH of the medium was 7.2–7.3. The medium was reduced with sodium sulfide and cysteine hydrochloride at initial concentrations of 100 mg l⁻¹. Strains ALDC^T and Lake were isolated using decane and hexane as growth substrates, respectively. Decane (5–10 mM) and hexane (7–10 mM) were provided in excess. The vials were incubated inverted at 31 °C and growth was assessed by both microscopy and sulfate depletion. The highest dilution at which growth occurred was used for the next dilution series. After repeated serial dilutions, pure cultures that contained cells of a single morphotype were obtained.

Purity controls. Purity was checked by using phase-contrast microscopy and by inoculating the cultures in their usual cultivation medium containing glucose (10 mM), lactate (10 mM) and yeast extract (0.1%) instead of an n-alkane. The lack of growth in these incubations suggested that no contaminants were present. In addition, the purity of the cultures was assessed by denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993; Duncan *et al.*, 2003). Cultures that exhibited a single morphology and a single DGGE band were assumed to be pure and were selected for characterization. Pure cultures were maintained and characterized using the same medium as that used for isolation, with the respective alkanes as growth substrates.

Physiological characterization. Incubation conditions promoting optimal growth rates were monitored as a function of sulfate

consumption. Occasionally, growth was also followed as an increase in OD₆₀₀. Sulfate was analysed by ion chromatography as described by Caldwell *et al.* (1998). Optimal salinity was determined using the basal medium with NaCl concentrations varying from 0 to 80 g l⁻¹. The pH limits in the basal medium were adjusted with bicarbonate buffer (CO₂/HCO₃⁻). Growth at pH 4.5 and 5.5 was assayed in media containing the physiological buffers 20 mM homopipes [homopiperazine-*N,N'*-bis-2(ethanesulfonic acid); 20 mM, pK_a = 4.61] and 20 mM MES (20 mM; pK_a = 6.15), respectively.

Utilization of electron donors other than n-alkanes, as well as culture vitamin requirements, were tested under optimal conditions after at least three consecutive transfers. Water-soluble substrates were added from anoxic sterile stock solutions to provide the concentrations indicated below. Vitamins were filter-sterilized. Aromatic substrates and 4-methyloctanoic acid at the indicated concentrations were added neat. The polyaromatic substrates naphthalene and 2-methylnaphthalene were supplied as an overlay dissolved in 2,2,4,4,6,8,8-heptamethylnonane as a 10 mg ml⁻¹ carrier. The ability to reduce electron acceptors was tested in basal seawater medium with decane as an electron donor, but without sulfate. For iron reduction experiments, the same medium was used except that the reducing agent was omitted. Iron reduction was determined with ferrozine reagent as described by Lovley & Phillips (1986) with both ferric citrate and ferric hydroxide as a source of Fe(III). Thiosulfate and sulfite were used at concentrations of 10 and 5 mM, respectively. Elemental sulfur (0.97 g l⁻¹) was provided as polysulfide (Widdel & Pfennig, 1992). Reduction of elemental sulfur, sulfite and thiosulfate was determined by monitoring sulfide production. Sulfide was analysed according to Trüper & Schlegel (1964). All experiments were replicated and repeated.

The G+C content of the DNA was determined by using standard HPLC analysis (Mesbah *et al.*, 1989) at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Genomic DNA was isolated using an Easy-DNA kit (Invitrogen).

PCR amplification of the 16S rRNA gene and phylogenetic analyses.

Nearly full-length 16S rRNA genes (*Escherichia coli* positions 8–1492) were obtained from DNA purified from cells by amplification with 'universal' eubacterial primers targeting conserved regions and the cycling conditions described by Herrick *et al.* (1993). Amplified DNA was purified from primers and unincorporated nucleotides and concentrated using Millipore Ultrafree-MC 30,000 NMWL Filter Devices to a concentration of 20–100 ng µl⁻¹. Sequencing of the purified PCR products was performed at the University of Oklahoma DNA Sequencing Facility on an ABI model 377 automated sequencer, using Ampli-TaqFS DNA polymerase and fluorescence-labelled dNTPs in a cycle-sequencing kit (ABI Prism Dye Terminator kit; PE Applied Biosystems). Amplification primers and two internal primers (704f, 907r; Johnson, 1994) were employed for sequencing of nearly full-length 16S rRNA genes. SEQUENCHER (Gene Codes) was used to assemble the fragments. The assembled sequence was compared with those in GenBank using BLASTN (Altschul *et al.*, 1997; National Center for Biotechnology Information). Sequences from the BLASTN search that most closely matched the sequences of the clones and selected outgroup strains were aligned using CLUSTAL_X (v. 1.81) (Thompson *et al.*, 1997). A dendrogram was constructed from the distance matrix using the neighbour-joining method in CLUSTAL_X and 1000 bootstrap replicates were performed to estimate the support for each branch (Felsenstein, 1985). Maximum-parsimony (1000 bootstrap replicates) and maximum-likelihood (100 bootstrap replicates) were used to confirm the phylogenetic placement of the cloned sequences (PAUP v. 4.0b10; Swofford, 2002).

RESULTS AND DISCUSSION

Repeated dilution series in liquid medium with decane or hexane as a growth substrate yielded two pure cultures of sulfate-reducing bacteria. The strains that were tentatively named ALDC^T and Lake originated from different sources (see Methods) and were both able to degrade n-alkanes completely to CO₂ with sulfate as an electron acceptor. The stoichiometry of alkane oxidation and sulfate reduction in the pure cultures was the same as that described previously for hydrocarbon mineralization by a highly purified enrichment (Kropp *et al.*, 2000). Thus, 20.3 mM sulfate was consumed for 3 mM decane metabolized. This is 87 % of that theoretically expected according to the equation:

No acetate production was found with strain ALDC^T or Lake during or after growth on hydrocarbons. The strains activated both protonated and deuterated n-alkanes by addition of the parent hydrocarbon to the double bond of fumarate (not shown) as described previously (Kropp *et al.*, 2000; Davidova *et al.*, 2005). Cells of strains ALDC^T and Lake had similar morphologies and phylogenetic analysis showed that the organisms were closely related (see below). We focused on strain ALDC^T for more detailed characterization.

Morphology

Cells of strain ALDC^T stained Gram-negative and were slightly curved, short rods with oval ends (2.5–3.0 × 1.0–1.4 µm), often occurring in pairs. They tended to form aggregates or large clusters (Fig. 1a, b). Rod-shaped cells were more common at the exponential growth phase, whereas late cultures exhibited bigger and more round cells. The cells were non-motile and did not form endospores. Bacterial growth was often observed as a thin layer floating in the upper part of the aqueous phase adhering to the overlying hydrocarbon layer. Cell buoyancy may have been regulated by the gas vesicles that were observed under phase-contrast microscopy as refractile bodies in the middle of the cells. Gas vesicles have been reported for other hydrocarbon-degrading, sulfate-reducing bacteria (Kniemeyer *et al.*, 2003).

Physiology

Strain ALDC^T was mesophilic, growing at temperatures of 17–50 °C with optimum growth occurring at 31–37 °C. The organisms also grew at pH values ranging from 4.5 to 8.2, with optimum growth at pH 6.5–7.2. NaCl was not required for growth, but salt concentrations up to 55 g l⁻¹ could be tolerated. The growth rate of strain ALDC^T remained optimal at salt concentrations ranging from 1.7 to 36 g l⁻¹. Strain ALDC^T could metabolize C₆ to C₁₂ n-alkanes. The doubling time on C₁₀ was 5.3 days. Strain ALDC^T exhibited a narrow substrate range. Apart from n-alkanes, it could metabolize only pyruvate (10 mM),

Fig. 1. Phase-contrast micrographs of cells (a) and cell aggregate (b) of strain ALDC^T. Bars, 10 µm.

butyrate (10 mM), hexanoic acid (2–5 mM) and 4-methyloctanoic acid (1.6 mM). It did not grow on H₂/CO₂ (20:80), acetate (2 mM), propionate (10 mM), fumarate (5 mM), succinate (5 mM), lactate (10 mM), citrate (10 mM), benzoate (3 mM), glucose (10 mM) or fructose (10 mM). It did not grow with the BTEX aromatic hydrocarbons benzene (350 µM), toluene (294 µM), ethylbenzene (250 µM) and *m*-xylene (250 µM), or 1-phenylhexane (166 µM) or 1-phenyldecane (125 µM). Strain ALDC^T did not use the polyaromatic hydrocarbons naphthalene and 2-methylnaphthalene. The inability of the culture to grow on BTEX hydrocarbons or on phenylalkanes was not a function of substrate toxicity. This was tested in separate incubations, in which the same amount of aromatic compound was added in combination with the usual amount of decane and no inhibition of growth was observed.

In addition to sulfate, strain ALDC^T could use thiosulfate (10 mM) as an electron acceptor for alkane degradation. Sulfite, elemental sulfur and Fe(III) were not reduced. Strain ALDC^T could grow in the basal medium without a vitamin supplement. However, folic acid, cyanocobalamin and thiamine improved growth, as did yeast extract (0.005 %). The DNA G + C content determined by HPLC was 53.6 mol%.

Phylogenetic analysis

Analysis of an almost-complete sequence (1495 bp) of the 16S rRNA gene of strain ALDC^T (GenBank accession no. DQ303457) and an almost-complete sequence (1484 bp) of the same gene of strain Lake (DQ303458) revealed that they belonged to the *Deltaproteobacteria* and were phylogenetically close to each other but not to other known mesophilic, alkane-degrading strains (AK-01, Pnd3, Hxd3 and CV2803^T) in the genus *Desulfatibacillum* (Cravo-Laureau *et al.*, 2004) (Fig. 2). Strains ALDC^T and Lake were phylogenetically very closely related (1483/1484 bp; 99.9% 16S rRNA gene sequence similarity). The 16S rRNA gene sequence similarity to other alkane-degraders that are members of the genus *Desulfatibacillum* was 88–87%. The novel strains were most closely related to *Desulfacinum* species (93–94.7% similarity) and *Syntrophobacter* species (91.7–94% similarity). Maximum-parsimony and maximum-likelihood methods also strongly supported (100%) a clade consisting of strains ALDC^T and Lake, with *Syntrophobacter* and *Desulfacinum* as sister clades.

The phenotypic characteristics of strains ALDC^T and Lake and members of the closely related genera *Desulfacinum* and *Syntrophobacter* were strikingly different. Thus, all known representatives of the genus *Desulfacinum* (*Desulfacinum infernum*, '*Desulfacinum subterraneum*', *Desulfacinum hydrothermale*) are thermophilic, sulfate-reducing bacteria that were isolated from high-temperature locations (Rees *et al.*, 1995; Sievert & Kuever, 2000; Rozanova *et al.*, 2001). They can use a broad range of substrates and are able to grow autotrophically with H₂. They are capable of growing with long-chain fatty acids. However, the ability to grow with alkanes has never been reported. The genus *Syntrophobacter* is composed of syntrophic bacteria that can grow by the oxidation of propionate in co-cultures with a H₂/formate-utilizing partner. They do not oxidize acetate or other fatty acids. They can grow slowly as pure sulfate-reducing cultures with certain substrates but, to our knowledge, the ability to grow with alkanes either in pure culture or in syntrophic associations has not been reported (McInerney *et al.*, 2005).

Fig. 2. Phylogenetic relationship of the 16S rRNA gene sequence of strain ALDC^T with those of its relatives and other alkane-degrading sulfate-reducing eubacteria. The tree was based on approximately 1340 bp and was constructed using the neighbour-joining algorithm. Bootstrap values (percentages of 1000 resamplings) greater than 80% are shown. Bar, 0.02 nucleotide substitutions per nucleotide.

The nucleotide sequence difference between strains ALDC^T and Lake and the aforementioned taxa is close to the accepted hypothetical sequence similarity value (95–93 %) for differentiating between genera (Fry *et al.*, 1991; Mullins *et al.*, 1995; Sheridan *et al.*, 2003). This distinction, in combination with the physiological differences and the fact that no members of *Desulfacinum* or *Syntrophobacter* have been reported to degrade n-alkanes, argues against the allocation of strain ALDC^T within either of these genera. Based on physiological characteristics and phylogenetic relationships with other *Deltaproteobacteria*, we propose that strain ALDC^T represents a novel species in a new genus, *Desulfoglaeba alkanexedens* gen. nov., sp. nov. Strain Lake, which is closely related to strain ALDC^T but was isolated from a different location, may represent another member of this new genus. Additional physiological details are required for accurate determination of its status.

Description of *Desulfoglaeba* gen. nov.

Desulfoglaeba (De.sul.fo.glae'ba. L. prep. *de* from; N.L. *sulfo* used for fem. n. *sulfas* sulfate in genus names of sulfate-reducing prokaryotes; L. fem. n. *glæba* clump/crumb/aggregate; N.L. fem. n. *Desulfoglaeba* sulfate-reducing clump/aggregate).

Mesophilic, sulfate-reducing bacteria that tend to form clusters. Cells are oval rods with refractile cores. Non-spore-forming. Strictly specialized in their substrate specificity. Oxidize alkanes completely. The type species is *Desulfoglaeba alkanexedens*.

Description of *Desulfoglaeba alkanexedens* sp. nov.

Desulfoglaeba alkanexedens (al.kan.ex.e'dens. N.L. n. *alkanum* alkane; L. part. adj. *exedens* eating up; N.L. part. adj. *alkanexedens* eating up alkanes).

Exhibits the following properties in addition to those given in the genus description. Cells are slightly curved, short rods with oval ends (2.5–3.0 × 1.0–1.4 µm), often occurring in pairs. Tends to form aggregates or large clusters. Growth occurs at 17–50 °C (optimum 31–37 °C) and pH 4.5–8.2 (optimum 6.5–7.2). NaCl is not required for growth but salt concentrations up to 55 g l⁻¹ can be tolerated. Sulfate and thiosulfate are used as electron acceptors. Alkanes (C₆–C₁₂), pyruvate, butyrate, hexanoic acid and 4-methyloctanoic acid can be used as electron donors. Vitamins are not required, but folic acid, cyanocobalamin (vitamin B₁₂) and thiamine improve growth. Yeast extract in low concentrations (0.005 %) stimulates growth.

The DNA G + C content of the type strain is 53.6 mol%. The type strain, ALDC^T (= JCM 13588^T = ATCC BAA-1302^T), was isolated from oily sludge collected from a naval wastewater-storage facility at the US Navy Craney Island Fuel Depot in Portsmouth, VA, USA.

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