Desulfovibrio alaskensis sp. nov., a sulphate-reducing bacterium from a soured oil reservoir

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A novel sulphate-reducing bacterium (Al1^T) was recovered from a soured oil well in Purdu Bay, Alaska. Light and atomic force microscopy observations revealed that cells were Gram-negative, vibrio-shaped and motile by means of a single polar flagellum. The carbon and energy sources used by the isolate and the salinity, temperature and pH ranges facilitating its growth proved to be typical of a partial lactate-oxidizing, moderately halophilic, mesophilic, sulphate-reducing bacterium. Analysis of the fatty acid profile revealed that $C_{18:0}$, iso$C_{15:0}$ and iso$C_{17:1}\omega 7c$ were the predominant species. Fatty acid profile and complete 16S rRNA gene sequencing demonstrated the similarity between strain Al1^T and members of the genus *Desulfovibrio*. The position of strain Al1^T within the phylogenetic tree indicated that it clustered closely with *Desulfovibrio vietnamensis* DSM 10520^T (98·9 % sequence similarity), a strain recovered from a similar habitat. However, whole-cell protein profiles, Fourier-transform infrared studies and DNA–DNA hybridization demonstrated that, in spite of the high level of 16S rRNA gene sequence similarity, there is sufficient dissimilarity at the DNA sequence level between *D. vietnamensis* DSM 10520^T and strain Al1^T (10·2 % similarity) to propose that strain Al1^T belongs to a separate species within the genus *Desulfovibrio*. Based on the results obtained, the name *Desulfovibrio alaskensis* sp. nov. is therefore proposed, with Al1^T (=NCIMB 13491^T=DSM 16109^T) as the type strain.

In offshore oil recovery processes, reservoir pressure is often maintained by the injection of a large volume of filtered sea water into the well. Indigenous bacteria from oil-bearing strata can therefore be introduced into the well. A sulphate-reducing bacterium (SRB) strain, previously referred to as Al1 (Beech & Cheung, 1995; Beech *et al.*, 1994; Zinkevich *et al.*, 1996), was isolated from material collected by E. van der Vende from a soured oil reservoir in Alaska (March 1991), a habitat with direct links to the marine environment, as the sea water from Purdu Bay was used in a secondary oil recovery system.

SRB enrichment was carried out using lactate as carbon source in marine Postgate medium B (Postgate, 1984) and purification was completed on semi-solid marine Postgate medium E (Postgate, 1984) as described elsewhere (Zinkevich *et al.*, 1996). Cultures were maintained anaerobically at 37 °C as stationary batch cultures in marine Postgate medium C (Postgate, 1984).

Light microscopy and atomic force microscopy (AFM) were used to study cell morphology. A Leitz light microscope (Laborlux S) was used to determine the Gram reaction (Gregersen, 1978), as well as cell shape and motility.

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Abbreviations: AFM, atomic force microscopy; FT-IR spectroscopy, Fourier-transform infrared spectroscopy; SRB, sulphate-reducing bacterium.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Al1^T is Y11984.

The fatty acid profile and an AFM image of strain Al1^T, FT-IR spectra of various SRB and a dendrogram based on these spectra are available as supplementary material in IJSEM Online.

AFM imaging was conducted in a Discoverer TMX2000 SPM (Veeco Metrology Group) as described by Feio *et al.* (1998) and a micrograph is available as Supplementary Fig. A in IJSEM Online. The physiological characterization of isolate Al1^T included determination of the temperature, pH and salinity ranges that allowed bacterial growth. These parameters were evaluated by growing cells in marine Postgate medium B for a period of 28 days under a range of conditions. The ability of cells to use different carbon and energy sources and electron donors was also tested. Results of the morphological and physiological characterization are given in Table 1 and summarized in the species description.

Lipid extraction, esterification, fatty acid purification and quantification using GC were performed as described previously (Bligh & Dyer, 1959). Lipids from lyophilized cells (50–100 mg) were extracted following a modified Bligh–Dyer method (White *et al.*, 1979). The extracted lipids were fractionated into neutral lipids, glycolipids and polar lipids by silicic acid column chromatography using appropriate volumes of chloroform, acetone and methanol, respectively. Phospholipids were subjected to mild alkaline methanolysis and the resulting fatty acid methyl esters were purified by TLC, GC and GC-MS (Guezennec, 1991). The position and geometry of the double bond of each monounsaturated fatty acid were determined using dimethyl disulphide derivatives according to a procedure described previously (Nichols *et al.*, 1986; Guezennec, 1991). The fatty acid profile of strain Al1^T (Supplementary Table A) revealed considerable amounts of monounsaturated fatty acids (21·2 % total fatty acids), of which 10·4 % were iso$C_{17:1}\omega7c$, a specific biomarker for the genus *Desulfovibrio* (Vainshtein *et al.*, 1992).

SRB chromosomal DNA was obtained using the guanidine isothiocyanate method (Zinkevich & Beech, 2000) from cultures grown for 7 days at 37 °C in 10 ml marine Postgate medium B. 16S rRNA genes of purified genomic DNA were amplified by PCR using eubacterial universal primers (Lane, 1991). Appropriate PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned according to standard methods (Sambrook *et al.*, 1989) in pGEM-T Easy vector (Promega). Restriction enzymes used were obtained from New England Biolabs. *Escherichia coli* JM 109 (Promega; Messing *et al.*, 1981) was

Table 1. Comparison of the morphological and physiological properties of strain Al1^T and *Desulfovibrio vietnamensis* DSM 10520^T

Optimum values are shown in parentheses. −, No growth; +, good growth; (+), weak growth; ND, not determined. Cells of both strains are vibrio-shaped with single polar flagella. Cultures did not show any growth in the absence of carbon sources and neither strain required vitamin supplements for growth. Both strains were able to utilize lactate and pyruvate and neither of them was able to utilize benzoate, butyrate, propionate (10 mM), acetate or butanol as electron donors. Both strains produced desulfoviridin and utilized sulphate, sulphite and thiosulphate as electron acceptors with lactate as an energy and carbon source. Neither was able to utilize nitrate. Data for *D. vietnamensis* were taken from Nga *et al.* (1996).

Characteristic	Al1^T	*D. vietnamensis* DSM 10520^T
Cell size (μm)	0·5–1·2 × 1·0–5·0	0·8–1·0 × 2·5–3·0
Temperature range (°C)	10–45 (37)	12–45 (37)
Salinity range (%, w/v, NaCl)	0–10 (2·5)	0–10 (5)
pH range	6·5–8·5 (7·0)	5·0–10·0 (7·5)
G+C content (mol%)	64·1	60·6
Utilization of electron donors in the presence of 15 mM sulphate:*		
Succinate†	+	−
Palmitate (10 mM)	−	ND
Glutamate	−	ND
Glycerol (10 mM)	−	+
Ethanol	−	+
Fumarate	ND	+
Formate	ND	+
Malate	ND	+
Fermentation in the absence of sulphate:		
Ethanol	(+)	−
Butanol	(+)	−
Growth rate (μ_{max}, h^{-1})‡	0·133	ND

*Unless otherwise stated, electron donors were tested at a concentration of 30 mM. In the tests carried out by Nga *et al.* (1996), 10 mM was used.
†Blackening of the medium in three successive subcultures was considered a positive result.
‡Determined in Postgate medium C at 37 °C.

used as a host strain for molecular cloning. *E. coli* JM 109 was grown in LB medium (Sambrook *et al.*, 1989) and SOC medium (Promega) at 37 °C. The solid LB medium was supplemented with 100 μg ampicillin ml^{-1}, 100 μg X-Gal ml^{-1} and 0·5 mM IPTG. Recombinant plasmid DNA was purified using a Qiagen plasmid mini kit. Both strands of the purified plasmid DNA (after restriction analysis) were sequenced by Cambridge BioSciences, Cambridge, UK.

The 16S rRNA gene sequence from Al1^T was added to an alignment of about 15 000 homologous bacterial 16S rRNA gene sequences using the alignment tool of the ARB program package (Strunk *et al.*, 1999). Phylogenetic trees were constructed using subsets of data that included representative sequences of members of the *δ-Proteobacteria*. Only sequences with at least 1300 nt were used. Distance matrix and maximum-likelihood methods, as implemented in the programs PHYLIP (Felsenstein, 1993), ARB and FASTDNAML (Maidak *et al.*, 2000), were used.

The comparison between the 16S rRNA gene sequences of Al1^T and some SRB strains of the genus *Desulfovibrio* revealed sequence similarities above 86 % with the majority of the species used. However, the closest relatives to strain Al1^T were *Desulfovibrio acrylicus* DSM 10141^T (89·0 %), *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough ATCC 29579^T (89·4 %) and *Desulfovibrio vietnamensis* DSM 10520^T (98·9 %). This latter strain was recovered from the water phase of a crude oil storage tank of an offshore oil platform in Vietnam (Nga *et al.*, 1996). The constructed phylogenetic trees were in good agreement with previously published ones (Devereux *et al.*, 1990; Feio *et al.*, 1998, 2000). Al1^T and *Desulfovibrio vietnamensis* DSM 10520^T formed a group in a lineage with an origin very close to the base of the family '*Desulfovibrionaceae*' (Fig. 1). A 16S rRNA gene sequence similarity of 97 % is commonly considered as the upper limit for the definition of separate species (Stackebrandt & Goebel, 1994). Although more than 97 % similarity indicates that strains may belong to the same species, it is now generally acknowledged that this rule does not always apply. DNA–DNA analysis was therefore performed to determine whether or not *Desulfovibrio vietnamensis* DSM 10520^T and Al1^T were sufficiently dissimilar for the latter to be considered to belong to a novel species.

Spectroscopic DNA–DNA hybridization of *Desulfovibrio vietnamensis* DSM 10520^T and strain Al1^T was undertaken by DSMZ (Braunschweig, Germany). DNA was isolated from bacterial cells by chromatography on hydroxyapatite according to the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with modifications reported by Huß *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992). In spite of the high similarity between strain Al1^T and *Desulfovibrio vietnamensis* DSM

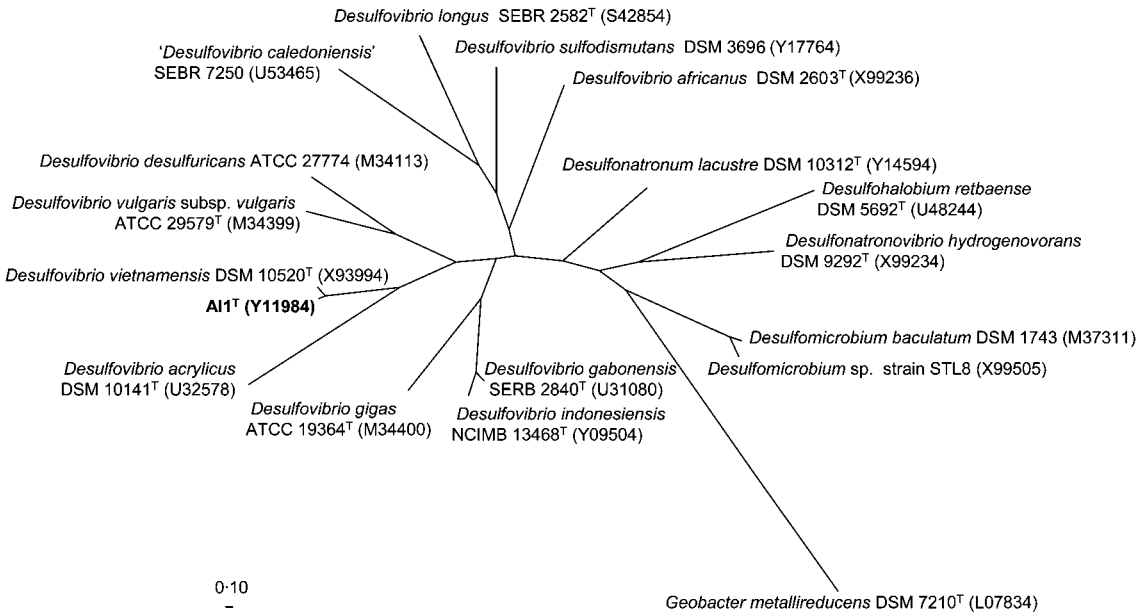

Fig. 1. Reconstructed phylogenetic tree based on 16S rRNA gene sequence relationships of strain A11^T and a selection of species from the *δ-Proteobacteria*. The tree is based on results of distance matrix analysis including complete or almost complete 16S rRNA gene sequences from representative bacteria of this subclass. The topology of the tree was corrected according to distance-matrix, maximum-parsimony and maximum-likelihood analyses of various datasets. Topologies that could not be resolved unambiguously are shown as multifurcation branching patterns, as previously recommended (Ludwig *et al.*, 1998). Accession numbers are given in parentheses.

10520^T observed at the 16S rRNA level, DNA–DNA hybridization revealed only 10·2 % relatedness. This result confirmed that the two strains are not related at the species level when the threshold value of 70 % for the definition of species is considered (Wayne *et al.*, 1987). Furthermore, the observed difference in melting temperatures between the DNA of the two strains (11 °C) indicates considerable difference in their DNA base composition, further confirming that strain Al1^T belongs to a novel species.

To further distinguish *Desulfovibrio vietnamensis* DSM 10520^T and Al1^T at the phenotypic level, whole-cell protein profiles and Fourier-transform infrared (FT-IR) spectroscopy analysis were performed. SDS-PAGE of whole-cell proteins is a rapid method for distinguishing bacterial species and has a similar level of discrimination to DNA–DNA hybridization (Jackman, 1987). Although there is not a genus-specific pattern (Jackman, 1987), differences in the protein patterns of whole cells reflect differences in the genomic content of the organism. Therefore, bacterial cells that are grown and recovered in an identical manner generate reproducible protein patterns, which can be used as fingerprints for their identification.

Whole-cell protein profile comparisons between *Desulfovibrio vietnamensis* DSM 10520^T and strain Al1^T were carried out with cultures grown under identical conditions in marine Postgate medium C for 5 days. Cells were harvested from 10 l batch cultures by centrifugation at 3000 ***g*** for 30 min. The pelleted cells were washed in 30 ml cold 50 mM MOPS buffer (pH 7·4) with 0·15 M NaCl and the pellet, after subsequent centrifugation at 3000 ***g*** for 30 min, was resuspended in 30 ml MOPS buffer. The cell preparation was then sonicated in a Soniprep 150 sonicator for 10 × 1 min bursts at 16 μm amplitude with 30 s intervals. Any unbroken cells and remaining culture debris were then removed by centrifugation at 3500 ***g*** for 30 min and the supernatant was stored at −20 °C for whole-cell protein profile analysis. Protein profile analysis was performed by SDS-PAGE in 12·5 % T acrylamide gels according to the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250 (Sigma). The protein profiles obtained for the whole cells of Al1^T and *Desulfovibrio vietnamensis* DSM 10520^T (Fig. 2) clearly demonstrated dissimilarities, thus supporting the evidence that strain Al1^T belongs to a novel species.

FT-IR spectroscopy provides chemical information about the biomolecular composition of whole bacterial cells. This technique is suitable for bacterial characterization due to the high specificity of obtained spectra (Schmitt *et al.*, 1995; Schmitt & Flemming, 1998). It can also be used to discern different bacterial species or even strains, providing cultures are grown under identical conditions. Hence, three independent replicate cultures of the SRB species investigated were grown anaerobically in 10 ml vials in Postgate medium C at 37 °C. Cells were harvested after 2 days incubation by centrifugation at 5000 ***g***. Pelleted cells were freeze-dried after washing with 0·9 % (w/v) NaCl solution.

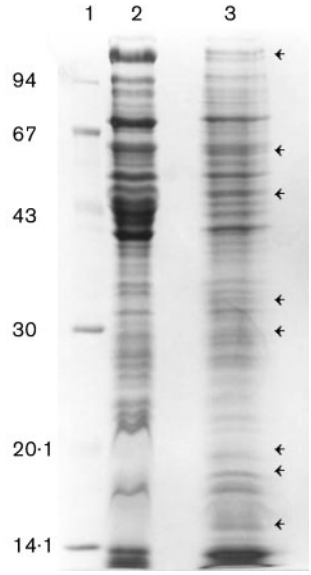

Fig. 2. Electrophoretogram showing whole-cell protein profiles of Al1^T (lane 2) and *D. vietnamensis* DSM 10520^T (lane 3); low molecular range protein markers (Pharmacia) are in lane 1. Loaded samples contained 100 μg protein per lane. Arrows indicate the positions of the protein bands with more significant differences.

Control replicates of sterile media were also lyophilized and analysed. Spectra were collected using a Mattson RS/2 research series spectrometer (ThermoUnicam) and data were manipulated using WINFIRST software. All spectra were acquired in transmission mode, by the KBr disc method. In each case, cells (2 mg) were diluted in 200 mg KBr powder to achieve a 1 % (w/w) concentration before pressing the disc. After a spectral quality check, data treatment consisted of vector-normalization of the spectra derivatives for statistical evaluation and construction of dendrograms.

The FT-IR spectra of Al1^T, *Desulfovibrio vietnamensis* DSM 10520^T, *Desulfovibrio indonesiensis* NCIMB 13468^T, *Desulfovibrio gabonensis* DSM 10636^T, *Desulfovibrio gigas* ATCC 19364^T, *Desulfovibrio desulfuricans* ATCC 27774, *Desulfovibrio vulgaris* subs. *vulgaris* strain Hildenborough ATCC 29579^T and *Desulfovibrio vulgaris* strain Woolwich NCIMB 8457 revealed considerable differences, mainly in the region between 1200 and 900 cm^{-1} (Supplementary Fig. B). This region is characterized by the presence of strain-specific bands that derive predominantly from the -C-O, -C-OH, -C-O-C and -C-O-P stretching vibrations. Statistical cluster analysis of the obtained FT-IR spectra, based on the bands at 1311 cm^{-1}, the phosphate groups with a maximum at 1234 cm^{-1} and the -C-O, -C-O-C and -C-O-H stretching region with bands at 1160 cm^{-1}, 1083 cm^{-1} and 969 cm^{-1}, led to the construction of a dendrogram (Supplementary Fig. C). Despite the fact that the dendrogram reflects whole cell composition, it shows a remarkable agreement with phylogenetic trees constructed based on full 16S rRNA

gene sequences. This analysis confirmed the high degree of similarity between strain Al1^T and *Desulfovibrio vietnamensis* DSM 10520^T but clearly established that they are separate species.

Desulfovibrio gigas ATCC 19364^T, *Desulfovibrio gabonensis* DSM 10636^T and *Desulfovibrio indonesiensis* NCIMB 13468^T formed a separate group in FT-IR analysis. This grouping was based on the similarity of these strains in the region between 1200 and 900 cm^{-1}, with a multiple band with peaks at 1128 cm^{-1}, 1083 cm^{-1} and 1046 cm^{-1}. Previous studies that did not include FT-IR analysis (Feio *et al.*, 1998) placed these three *Desulfovibrio* strains in the same group, thus verifying the FT-IR data and validating the use of FT-IR spectroscopy of whole cells as a rapid and highly sensitive technique for identification and characterization of SRB.

Despite the high level of similarity found between the 16S rRNA gene sequences of strain Al1^T and *Desulfovibrio vietnamensis* DSM 10520^T and the similarities in the environment from which the two isolates were recovered, the remaining evidence, i.e. DNA–DNA hybridization, FT-IR analysis and whole-cell protein profiles, clearly demonstrates the difference between these two strains. Our data strongly indicate that strain Al1^T represents a novel species belonging to the genus *Desulfovibrio* and classification of this isolate as a representative of a novel species, *Desulfovibrio alaskensis* sp. nov., is therefore proposed.

Description of *Desulfovibrio alaskensis* sp. nov.

Desulfovibrio alaskensis (al.ask.en′sis. N.L. masc. adj. *alaskensis* from Alaska, referring to the place of isolation).

Gram-negative, non-spore-forming, vibrio-shaped cells, 1·0–5·0 × 0·5–1·2 μm. Cells occur singly and are motile by means of a single polar flagellum. Grows at pH 6·5–8·5, 10–45 °C and in 0–10 % (w/v) NaCl. Maximum growth rate under optimal growth conditions in marine Postgate medium C [37 °C, pH 7·0 and 2·5 % (w/v) NaCl] using lactate as carbon source is 0·133 h^{-1}. Vitamins are not required for growth. Strictly anaerobic, reduces sulphate, sulphite and thiosulphate, producing sulphide. Nitrate is not used as an electron acceptor. Substrates that are oxidized by sulphate reduction are lactate, pyruvate and succinate. Ethanol and butanol can be utilized fermentatively (for a limited number of generations). Desulfoviridin-type sulphite reductase is present. DNA G+C content is 64·1 mol%. Major cellular fatty acids are $C_{18:0}$, $isoC_{15:0}$ and $isoC_{17:1}\omega7c$.

The type strain is Al1^T (=NCIMB 13491^T=DSM 16109^T), isolated from the production fluids of offshore oilfields in Alaska.

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