Desulfovibrio alaskensis sp. nov., A SULPHATE REDUCING BACTERIUM FROM A SOURED OIL RESERVOIR

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RUNNING TITLE:

Characterisation of a novel sulphate-reducing bacterium

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

SUMMARY

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, rod-shaped and motile. Physiological characterisation, analysis of the fatty acid profile and complete 16S rRNA sequencing demonstrated a strong similarity between the new species and members of the *Desulfovibrio* genus. The position of the Al1^T strain within the phylogenetic tree indicated that it clustered closely with *Desulfovibrio vietnamensis*. Whole-cell protein profiles, FT-IR studies and DNA-DNA hybridisation demonstrated that, in spite of the high level of 16S rRNA similarity, there is sufficient dissimilarity at DNA level between *D. vietnamensis* and Al1^T strains to consider the isolate a new separate species within the genus *Desulfovibrio*. Based on the obtained results, the name *Desulfovibrio alaskensis* sp. nov. is therefore proposed, with the type strain Al1^T (NCIMB 13491^T= DSMxxx^T).

INTRODUCTION

Although the proliferation of sulphate-reducing bacteria (SRB) in oil-bearing formations has been demonstrated as responsible for oil well souring (Leu *et al*, 1998) the mechanisms of the process are not fully understood. Little is known about the ecology of SRB communities in oil-rich formations hence, the study of SRB associated with oil-bearing strata would provide a better understanding of the ecology of these organisms and thus help to elucidate the souring process. Our investigation was undertaken to characterise and subsequently identify a sulphate-reducing bacterium isolated from a severely soured oil reservoir in Purdu Bay, Alaska, in order to compare this isolate with other strains recovered from similar habitats found in geographically distant areas.

METHODS

Organisms and culture conditions

The SRB strain previously referred to as $Al1^T$ (Beech & Cheung, 1995; Beech *et al*, 1994; Zinkevich *et al*, 1996) was recovered in March 1991 from a soured oil reservoir in Purdu Bay, Alaska by Dr. Ewout van der Vende.

The 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 inspected for aerobic and anaerobic contaminants using the spread plate technique on solid Nutrient and Anaerobic agars (Difco, UK). The cells were grown anaerobically at 37 ºC as stationary batch cultures in marine Postgate medium C (Postgate, 1984). Culture tubes (10 ml) were filled with an appropriate culture medium, purged with a N_2 flux to create anaerobiosis and autoclaved for 30 min at 121 °C. For large-scale growth, 1 to 10 l screw-cap bottles were autoclaved and degassed with filter-sterilised oxygen-free nitrogen prior to inoculation. In all experiments, a 10 % (v/v) inoculum was used and cultures were incubated at 37 ºC. Stock cultures were stored at 4 ºC and transferred at weekly intervals to fresh medium to maintain viability.

Desulfovibrio vietnamensis DSM 10520 was purchased from DSMZ (Braunschweig, Germany). Other SRB strains used for the study *i.e. Desulfovibrio indonesiensis* NCIMB 13468, *Desulfovibrio gabonensis* DSM 10636, *Desulfovibrio gigas* ATCC 29494, *Desulfovibrio desulfuricans* ATCC 27774, *Desulfovibrio vulgaris* ATCC 29579 and *Desulfovibrio vulgaris* Woolwich NCIMB 8457 were available from our own culture collection.

Morphological characterisation

Light microscopy and Atomic Force Microscopy (AFM) were employed 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.

To facilitate AFM observations, a few drops of a 3 day old bacterial culture grown in marine Postgate medium C were deposited on a 1 cm^2 glass slide and air-dried. The cells were heat-fixed by passing over a flame. Fixed samples were then washed with distilled water to remove any salts present and dried in air under ambient conditions. AFM imaging was conducted in a Discoverer TopoMetrix TMX2000 SPM (Veeco Metrology Group, Santa Barbara, California), equipped with a scanner capable of a maximum *x-y* translation of 70 μm x 70 μm. The microscope was operated in contact mode using standard silicon nitride tips integrated on cantilevers of 0.036 Nm^{-1} force-constant.

Physiological studies

The physiological characterisation of the $A11^T$ isolate included the 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. To determine the ability of the cells to utilise different carbon and energy sources, bacteria were grown in medium where sodium lactate had been replaced with formate, glutamate, pyruvate, succinate, palmitate, acetate, propionate, butyrate, benzoate (as the sodium salts of their respective acids) as well as ethanol, butanol and glycerol, all at a concentration of 30 mM. Blackening of the media in three successive subcultures was considered a positive result.

Lipid analysis

Lipid extraction, esterification, fatty acid purification and quantification using gas chromatography (GC) were performed as previously described (Bligh & Dyer, 1959). Lipids from lyophilised cells (50 to 100 mg) were extracted following a modified Bligh-Dyer method (Bligh & Dyer, 1959; 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. The phospholipids were subjected to mild alkaline methanolysis, and the resulting fatty acid methyl esters purified by thin-layer chromatography, GC and GC-mass spectrometry (GC-MS) (Guezennec, 1991). The position and geometry of the double bond of each monosaturated fatty acid were determined using the dimethyl disulphide derivatives according to procedure described previously (Nichols *et al*, 1986, Guezennec, 1991).

PCR amplification and cloning of 16S RNA genes

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. 16Sr RNA genes of purified genomic DNA were amplified by PCR using eubacterial universal primers (Lane, 1991). PCR was performed in 50 µl reaction mixtures, containing 1 unit of Taq polymerase, 5 µl of 10 x concentrated Taq PCR buffer, 2.5 mM Mg^{2+}, 0.6 µM of each primer and 200 µM each dATP, dCTP, dGTP, dTTP (Boehringer Mannheim). Initial denaturation of the template DNA was completed at 95 °C for 5 min and 25 amplification cycles were performed according to the following scheme: denaturation at 95 °C for 1 min; annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. The final PCR step was carried out at 72 °C for 10 min. Ten per cent of the reaction volume was analysed by 0.8% (w/v) agarose gel electrophoresis (Sigma, Type II, medium EEO) in TAE buffer (National Diagnostics) (Sambrook *et al*, 1989). The 1 kb DNA ladder (Gibco BRL) was used as a standard.

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 (Technical manual, Promega). Restriction enzymes used were obtained from New England Biolabs. *Escherichia coli* JM 109

(Promega) (Messing *et al*, 1981) was used as a host strain for molecular cloning. *E. coli* JM 109 strain was grown in LB medium (Sambrook *et al*, 1989) and SOC medium (Promega, technical manual 042) 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. Recombinat plasmid DNA was purified using QUIAGEN plasmid mini kit (QUIAGEN).

Both strands of the purified plasmid DNA (after restriction analysis) were sequenced by Cambridge BioSciences (UK).

16S rRNA sequence alignment

The 16S rRNA gene sequence from $Al1^T$ was added to an alignment of about 15000 homologous bacterial 16S rRNA sequences using the alignment tool of the ARB program package (Strunk *et al*, 1998-2000). Phylogenetic trees were constructed using subsets of data that included representative sequences of members of the δ-subclass of Proteobacteria. Only sequences with at least 1300 nucleotides 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.

DNA-DNA hybridisation

Spectroscopic DNA-DNA hybridisation of *Desulfovibrio vietnamensis* and Al 1^T isolate was performed by Dr. Peter Schumann of DSMZ in Braunschweig, Germany. DNA was isolated from bacterial cells by chromatography on hydroxyapatite according to the procedure of Cashion *et al.* (1977). DNA-DNA hybridisation 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 spectrophometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories Inc., Oberlin, Ohio, USA). Renaturation rates were computed with the TRANSFER.BAS programme (Jahnke, 1992).

Whole-cell protein profiles

Cells grown in marine Postgate medium C for 2-5 days were harvested from 10 l batch cultures by

centrifugation at 3000 g for 30 min. The pelleted cells were extensively washed in 30 ml cold 0.15 M NaCl/ 50 mM MOPS buffer pH 7.4, 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 x 1 min bursts at 16 μm amplitude with 30 sec 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 (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 (Sigma).

Fourier-Transform Infrared (FT-IR) Spectroscopy

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 of incubation by centrifugation at 5000 g. Pelleted cells were freeze-dried after extensive washing with 0.9% (w/v) NaCl solution. Control replicates of sterile media were also lyophilised and analysed.

Spectra were collected using a Mattson RS/2 research series spectrometer (ThemoUnicam, Cambridge, UK) and data manipulated using WinFIRST software. All spectra were acquired in transmission mode, by the KBr disc method. In each case, 2 mg of cells were diluted in 200 mg of KBr powder to achieve a 1% (w/w) concentration before pressing the disc.

After a spectral quality check, data treatment consisted of the vector-normalisation of the spectra derivatives for statistical evaluation and construction of dendograms.

RESULTS & DISCUSSION

Results of the morphological and physiological characterisation are given in the species description.

The fatty acid profile of the A11^T strain revealed large amounts of branched fatty acids, which account for

50.6% of the total fatty acids (results are available as supplementary data in IJSEB online). Considerable amounts of monounsaturated fatty acids were also found (21.2% o the total fatty acids). Of the former, 10.4% were iso $C_{17:1\omega7c}$, the specific biomarker for the genus *Desulfovibrio* (Vainshtein *et al*, 1992). The overall predominant species was $C_{18:0}$ (40.7%).

Genotypic Analysis

The full 16S rRNA sequence data analysis confirmed that the strain $Al1^T$ belongs to the genus *Desulfovibrio*. The comparison between the 16SrRNA sequences of $Al1^T$ and some SRB strains of the *Desulfovibrio* genus (available as supplementary data in IJSEB online) revealed a high level of homology (98%) with *D. vietnamensis*. This strain was recently isolated 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 (Devereaux *et al*, 1990; Feio *et al*, 1998; Feio *et al*, 2000). $Al1^T$ and *D. vietnamensis* form a group in a lineage with an origin very close to the base of the *Desulfovibrionacea* family (available as supplementary data in IJSEB online). A similarity of 97% of the 16S rRNA gene sequence 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 and that DNA analysis ought to be performed to confirm 16S rRNA results. In spite of the high similarity between $Al1^T$ isolate and *D. vietnamensis* observed at 16S rRNA level, DNA-DNA hybridisation revealed only 10.2% similarity. 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 indicates considerable difference in their DNA base composition, further confirming that $Al1^T$ strain is a novel species.

Whole-cell protein profiles and FT-IR spectroscopic analysis

SDS-PAGE of whole cell proteins is a rapid method for distinguishing bacterial species and has a similar level of discrimination to DNA: DNA hybridisation (Jackman, 1987). Bacterial cells that are grown under identical conditions produce reproducible protein patterns, which can be used as fingerprints for the

identification of the strains under investigation. This approach has been widely used in the systematics of numerous bacterial strains (Costas *et al*, 1993; Kersters & Deley, 1980) although it has had a limited application to sulphate-reducing bacteria (Feio *et al*, 1998; Nga *et al*, 1996). Usually, there is not a genus specific pattern (Jackman, 1987), however differences in the protein patterns of whole cells reflect differences in the genomic content of the organism. The protein profiles obtained for the whole cells of Al1^T and *D. vietnamensis* (available as supplementary data in IJSEB online) clearly demonstrated dissimilarities in their profiles, thus supporting the evidence that the former is a novel species.

FT-IR spectroscopy provides chemical information about the biomolecular composition of the intact bacterial cells. This approach is suitable for characterisation due to the high specificity of obtained spectra (Schmitt *et al*, 1995; Schmitt & Flemming, 1988). The technique can also be used to discern different bacterial strains or even substrains, providing cultures are grown under the same conditions. The FT-IR spectra of Al1^T, *Desulfovibrio indonesiensis* NCIMB 13468, *Desulfovibrio gabonensis* DSM 10636, *Desulfovibrio gigas* ATCC 29494, *Desulfovibrio desulfuricans* ATCC 27774, *Desulfovibrio vulgaris* ATCC 29579 and *Desulfovibrio vulgaris* Woolwich NCIMB 8457 revealed considerable differences which are mainly noted in the region between 1200 cm^{-1} and 900 cm^{-1} (Figure 1). This region is characterised by the presence of strain specific bands which predominantly derive 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, the polysaccharides with bands at 1160 cm^{-1}, 1083 cm^{-1} and 969 cm^{-}1, led to the construction of dendograms, which showed a remarkable agreement with the phylogenetic trees constructed using full 16S rRNA gene sequences (Figure 2). This analysis confirmed the high degree of similarity between strain Al1^T and *D. vietnamensis*.

The strains *D. gigas, D. gabonensis* and *D. indonesiensis* form a separate group. This different grouping refers to a similarity of these latter strains in the region between 1200 cm^{-1} and 900 cm^{-1} with a multiple band with peaks at 1128 cm^{-1}, 1083 cm^{-1} and 1046 cm^{-1}. A band at 794 cm^{-1} in the fingerprint region of *D. gabonensis* is unique to this isolate. Previous studies which did not include FT-IR approach (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 the discrimination and characterisation of SRB.

Protein analysis

The purification and characterisation of key enzymes involved in the sulphate metabolism of $Al1^T$ has already been accomplished (data not shown). The study confirmed the presence of a periplasmic and cytoplasmic hydrogenase system and desulfoviridin-type sulphite reductase, thus providing further evidence that $Al1^T$ belongs to the genus *Desulfovibrio*. The results obtained from the spectroscopic characterisation of the cytochrome c_3 revealed very distinct features from those of other tetrahemic cytochromes described to date for SRB. Advanced NMR studies and further biochemical analysis aiming to correlate the differences found in the structure of the proteins from $Al1^T$ and their taxonomic significance are currently in progress.

CONCLUSIONS

In offshore oil recovery processes, reservoir pressure if often maintained by the injection of a large volume of filtered seawater into the well. Indigenous bacteria from oil-bearing strata can therefore be introduced into the well. Strain $Al1^T$ was recovered from a soured oil reservoir in Alaska, a habitat with direct links to the marine environment as the seawater from Purdu Bay was used in a secondary oil recovery system. Morphological and physiological characterisation of the strain indicated that it was a likely member of the *Desulfovibrio* genus. 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 oxidiser, moderately halophilic, mesophilic sulphate-reducing bacterium. Despite the high level of homology found between 16S rRNA sequences of $Al1^T$ isolate and *Desulfovibrio vietnamensis* and the similarities in the environment from which the two isolates were recovered, the remaining evidence, *i.e.*, DNA-DNA hybridisation, FT-IR analysis, fatty acid analysis and whole-cell protein profiles, clearly demonstrate the difference between these two strains. Our data strongly supports the statement that the $Al1^T$ isolate is a novel species belonging to the *Desulfovibrio* genus and the classification of this isolate

as *Desulfovibrio alaskensis* sp. nov. is therefore proposed.

Description of *Desulfovibrio alaskensis* sp. nov.

Desulfovibrio alaskensis (alas-ken-sis, L. adj. *alaskensis*, from Alaska, referring to the place of isolation). Gram-negative, non-spore-forming rod-shaped cells ranging from 1 to 5 μm in length and 0.5 to 1.2 μm in width. Cells occurred singly and are motile by means of a single polar flagellum. The pH that allows growth ranges between 6.5 and 8.5. Temperature ranges between 10 and 45 °C and the salinity that facilitates growth ranges between 0 and 10% (w/v) NaCl. A maximum growth rate under optimal growth conditions in marine Postgate medium C (37 ºC, pH 7.0 and 2.5 % (w/v) NaCl) using lactate as carbon source was determined as 0.133 h^{-1}. Vitamins are not required for growth. Strictly anaerobic, reduces sulphate and sulphite producing sulphide. Nitrate is not used as electron acceptor. Substrates that are oxidised by sulphate reduction are lactate, pyruvate and succinate. Ethanol and butanol can be utilised (for a limited number of generations) when replacing sodium sulphate. Desulfoviridin-type sulphite reductase is present. Major cellular fatty acids are C18:0, iso C15:0 and iso C17:1ω7c. Isolated from the production fluids of offshore oil fields in Alaska. Type strain is A11^T (= NCIMB 13491^T=DSM xxx^T).

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Figure 1. FT-IR spectra of the fingerprint region of different SRB species belonging to the genus *Desulfovibrio* studied.

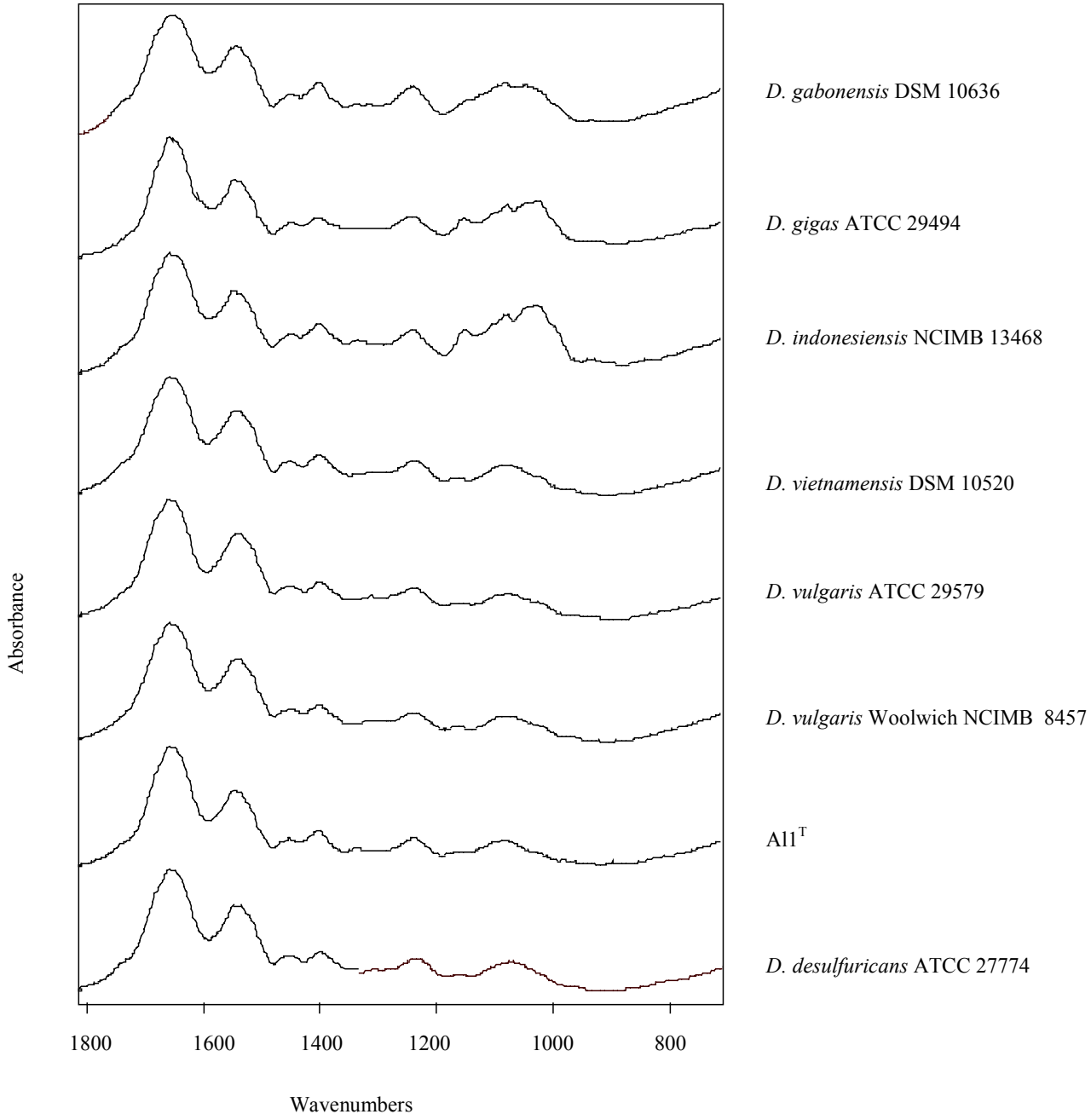

Figure 2. Classification of selected *Desulfovibrio* species based on statistical cluster analysis of their FT-IR spectra. The difference between the strains increases with increasing heterogeneity values.