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Desulfobacter psychrotolerans sp. nov., a new psychrotolerant sulfate-reducing bacterium and descriptions of its physiological response to temperature changes

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Abstract

A psychrotrolerant acetate-oxidizing sulfate-reducing bacterium (strain akvb^T) was isolated from sediment from the northern part of The North Sea with annual temperature fluctuations between 8 and 14 °C. Of the various substrates tested, strain akvb^T grew exclusively by the oxidation of acetate coupled to the reduction of sulfate. The cells were motile, thick rods with round ends and grew in dense aggregates. Strain akvb^T grew at temperatures ranging from −3.6 to 26.3 °C. Optimal growth was observed at 20 °C. The highest cell specific sulfate reduction rate of 6.2 fmol cell^{-1} d^{-1} determined by the $^{35}SO_4^{2-}$ method was measured at 26 °C. The temperature range of short-term sulfate reduction rates exceeded the temperature range of growth by 5 °C. The Arrhenius relationship for the temperature dependence of growth and sulfate reduction was linear, with two distinct slopes below the optimum temperatures of both processes. The critical temperature was 6.4 °C. The highest growth yield (4.3–4.5 g dry weight mol^{-1} acetate) was determined at temperatures between 5 and 15 °C. The cellular fatty acid composition was determined with cultures grown at 4 and 20 °C, respectively. The relative proportion of cellular unsaturated fatty acids (e.g. 16:1ω7c) was higher in cells grown at 4 °C than in cells grown at 20 °C. The physiological responses to temperature changes showed that strain akvb^T was well adapted to the temperature regime of the environment from which it was isolated. Phylogenetic analysis showed that strain akvb^T is closest related to *Desulfobacter hydrogenophilus*, with a 16S rRNA gene sequence similarity of 98.6%. DNA–DNA-hybridization showed a similarity of 32% between *D. hydrogenophilus* and strain akvb^T. Based on phenotypic and DNA-based characteristics we propose that strain akvb^T is a member of a new species, *Desulfobacter psychrotolerans* sp. nov.

Introduction

Acetate, which is the end product of numerous anaerobic fermentation pathways, is quantitatively one of the most important substrates available to sulfate-reducing bacteria (SRB) and may provide up to 50% of the reducing equivalents for sulfate reduction in marine sediments (Sørensen et al. 1981; Christensen 1984; Parkes et al. 1989). Despite the availability of acetate as a substrate for SRB in marine sediments, only a few authenticated SRB's are able to metabolize acetate (e.g. Widdel and Pfennig 1977, 1981; Brysch et al. 1987; Oude Elferink et al. 1995, 1999). One reason for this may be the low energy gain of the reaction (Rabus et al. 2000).

A nutritionally restricted group of sulfate reducers, preferentially using acetate as their electron donor and carbon source, constitutes the genus *Desulfobacter*. Currently six species have been validly described: *Desulfobacter postgatei* (Widdel and Pfennig 1981), *Desulfobacter hydrogenophilus*, *Desulfobacter curvatus*, *Desulfobacter latus* (Widdel 1987), *Desulfobacter vibrioformis* (Lien and Beeder 1997) and *Desulfobacter halotolerans* (Brandt and Ingvorsen 1997). These strains are mesophilic according to the definitions given by Morita (1975) and Russell (1990) (see below) except for *D. hydrogenophilus*, which is the only strain that is able to grow at temperatures as low as 0 °C and thus is psychrotolerant. Microorganisms adapted to low and temperate temperatures have been classified according to their temperature regimes for growth, as psychrophilic (from < 0 to ≤ 20 °C with optimal temperature ≤ 15 °C), psychrotolerant (from < 0 to ≤ 35 °C with optimal temperature ≤ 25 °C) and mesophilic (from > 10 to > 35 °C with optimal temperature > 25 °C) (Morita 1975; Russell 1990).

According to this definition psychrotolerant bacteria have a broader temperature spectrum than both psychrophilic and mesophilic bacteria. Consequently, psychrotolerant bacteria may inhabit both temperate and low temperature environments (Harder and Veldkamp 1968; Isaksen and Jørgensen 1996; Sass et al. 1998; Rabus et al. 2002). Microorganisms that can grow at low temperatures are of particular importance in marine ecosystems since the major part of the worlds oceans are permanently or seasonally cold (< 5 °C) (Russell 1990). Although sulfate reducers are responsible for up to 50% of the organic carbon remineralization in both temperate and low temperature marine sediments (Jørgensen 1982; Nedwell et al. 1993; Sagemann et al. 1998), only a few psychrophilic and psychrotolerant sulfate reducers have been isolated and characterized so far (Brysch et al. 1987; Isaksen and Jørgensen 1996; Isaksen and Teske 1996; Sass et al. 1998; Knoblauch and Jørgensen 1999; Knoblauch et al. 1999; Rabus et al. 2002).

Temperature has a major effect on metabolic rates as well as on growth rates (Russell and Hamamoto 1998). Arrhenius plots, generally used to evaluate the temperature dependence of chemical reactions, have been successfully used to investigate the temperature response of growth and respiration in pure cultures and microbial communities (Harder and Veldkamp 1971; Mohr and Krawiec 1980; Guillou and Guespin-Michel 1996; Isaksen and Jørgensen 1996; Sagemann et al. 1998; Rabus et al. 2002). The linearly descending portion of the Arrhenius plot corresponds to the physiological temperature range for growth and/or respiration. Deviation from linearity indicates that bacteria are under stress and can no longer maintain a maximal growth rate at a given temperature. Consequently, mesophilic, psychrotolerant and psychrophilic bacteria are, at low temperatures, differentiated by their ability to maintain potential maximum growth rates at a given temperature (Hébraud and Potier 1999).

In this communication, we report on the isolation and characterization of a psychrotolerant sulfate-reducing bacterium, strain akvb^T, belonging to the genus *Desulfobacter* that is able to grow below 0 °C. Strain akvb^T was enriched from North Sea sediment with acetate and sulfate as electron donor and electron acceptor, respectively. On the basis of DNA-based and phenotypic differences between strain akvb^T and validly described members of the genus *Desulfobacter*, the strain is proposed as the type strain of a new species, *Desulfobacter psychrotolerans* sp. nov.

Materials and methods

Source of organism

Strain akvb^T was isolated from surface sediment (0–1 cm) sampled at the location Kvitebjoern (61°10′41″ N, 2°14′51″ E) in the northern part of The North Sea during cruise 169 with R/V Heincke in May 2002. The sample site was a methane seep that was covered by patches of *Beggiatoa* spp. On an annual basis the bottom water temperature at the sampling site varies between 8 and 14 °C, due to the influence of the North Atlantic current (Bundesamt für Seeschiffahrt und Hydrographie 2004). The bottom water temperature at the time of sampling was 12 °C. The samples were transferred to glass bottles containing a defined CMS-medium supplemented with acetate (20 mM) and sulfate (20 mM) and incubated at 4 °C until they were transferred to the laboratory where the incubation was continued at 15 °C.

Enrichment, isolation and cultivation

For enrichment and isolation of acetate-oxidizing sulfate reducers a marine medium (CMS-medium) was prepared with acetate (20 mM) and sulfate (20 mM) as substrates. The medium contained (each g l^{-1}): NaCl, 20; KCl, 0.7; NH_4Cl, 0.2; KH_2PO_4, 0.2; Na_2SO_4, 3.0. The medium was autoclaved and afterwards allowed to cool to room temperature under a $N_2 : CO_2$ (9:1 (v/v)) atmosphere. Solutions of $CaCl_2 \cdot 2H_2O$ (1.5 g in 50 ml Milli-Q water), $MgCl_2 \cdot 6H_2O$ (10.6 g in 50 ml Milli-Q water) and $FeCl_2 \cdot 4H_2O$ (0.517 g in 10 ml HCl (10 mM)) were prepared under a N_2 atmosphere, autoclaved separately and added to 1 l of cold medium. In addition, $NaHCO_3$ (2.5 g in 30 ml Milli-Q water) autoclaved under CO_2 atmosphere was added to 1 l of medium as buffer. Vitamin solutions were prepared and added as described by Abildgaard et al. (2004). If necessary, pH was adjusted to 7.2–7.4 with sterile HCl solution (1 M). The medium was filled into 50 ml sterile screw-capped bottles. Acetate (20 mM in final concentration) was added from a sterile stock solution prior to inoculation. An inoculum size of 10% (w/v) was used and incubations were carried out at 15 °C in darkness, unless noted otherwise. Growth was examined by phase contrast microscopy. Although some ferrous sulphide was visible on inoculation, sulfate reduction was evident from the extensive formation of acid labile ferrous sulfide during incubation, to the extent that it covered the inside of the glass bottles as a shiny black film. Isolation of pure cultures was achieved by repeated application of the agar-shake dilution method (Widdel and Bak 1992).

Two additional media were used for the cultivation and characterization of the isolate: MV-medium was prepared according to the procedure followed during the preparation of the CMS-medium, except that $FeCl_2 \cdot 4H_2O$ was replaced by 1.5 ml of a 0.5 M $Na_2S \cdot 9H_2O$ (autoclaved under a N_2 atmosphere at 121 °C) as a reductant and $CaCl_2 \cdot 2H_2O$ (0.2 gl^{-1}) and $MgCl_2 \cdot 6H_2O$ (3 g l^{-1}) was added directly to the medium prior to autoclaving . DSMZ medium 195 (DSMZ 2005) with the following modification was used. The vitamin solution was replaced by the solutions used in the CMS medium described above. The DSMZ medium 195 was prepared according to the procedure used for the preparation of CMS-medium.

The purity of the cultures was repeatedly checked by microscopy. In addition the culture was transferred to a complex medium, which contained yeast extract (0.1% (w/v)), fumarate (10 mM), glucose (10 mM) and pyruvate (10 mM). Aerobic growth was tested on nutrient broth agar plates. No growth occurred at 5, 15 or 30 °C on these media.

Photomicrographs

The cellular morphology was examined routinely by phase-contrast microscopy. Photomicrographs were taken with an Apotome, epi-fluorescense microscope (Zeiss, Germany) on gelatine (1% (w/v)) coated slides.

Physiology and metabolism

A range of substrates were tested in duplicate 15 ml Hungate tubes with CMS- or MV-medium by substituting either acetate or sulfate with an alternative electron donor or electron acceptor under a gas phase containing a mix of oxygen-free N_2:CO_2 (9:1 (v/v)). Growth was determined microscopically by an increase in cell densities. Cultures with and without acetate or sulfate served as controls. All cultures were transferred to fresh medium containing a given substrate combination several times to confirm the results and maintained for 4–6 months and checked for growth on a regular basis.

The effect of temperature, NaCl concentrations and pH on growth was determined in MV- or DSMZ 195-medium supplemented with acetate (20 mM) and sulfate (20 mM). Since the culture formed aggregates during growth it was not possible to follow growth by optical density (OD) measurements. Growth was thus determined by following the consumption of sulfate and acetate during the incubations. Temperature experiments were carried out in duplicate in a temperature gradient block at 18 different temperatures ranging from −3.6 to 32.5 °C. Growth rates were calculated from the linear regression on ln([acetate]) data as a function of time. The effect of NaCl on growth was determined at 15 different NaCl concentrations ranging from 0 to 46 g l^{-1}. The tests were carried out in duplicate at 5 °C. The pH range was determined in triplicate cultures adjusted to pH values between 4.5 and 9.1. The

incubation temperature was 5 °C. The vitamin demand was tested for five subsequent transfers to medium without vitamins. Cell densities were determined microscopically.

Determination of the influence of temperature on growth yield

Growth yield was expressed as the ratio between cellular biomass produced and the amount of acetate consumed. The influence of temperature on the growth yield of strain akvb^T was studied with triplicate cultures grown in 100 ml screw-capped bottles containing MV-medium with acetate (20 mM) and sulfate (20 mM) at 0, 5, 10, 15 and 20 °C, respectively. Cultures were harvested immediately after inoculation and during the exponential growth phase by filtration on GF/F filters (GF-75, 25 mm, Advantec). The filters were washed with 100 µl HCl (50 mM) to dissolve the bicarbonate and 1 ml of milli-Q water and dried at room temperature in sterile Petri dishes. The dried filters were placed in tin capsules and the cell carbon content was determined with a NA 1500CN analyser (Carlo Erba, Italy). The carbon content was converted into dry weight biomass by multiplication by a factor of 2.1, thus assuming the overall cell composition of the cell organic matter is $C_4H_7O_3$ (Widdel and Pfennig 1981). The amount of acetate assimilated into cell material was calculated by the following equation:

$$17CH_3COO^- + 11H_2O \rightarrow 8(C_4H_7O_3) + 2HCO_3^- + 15OH^-,$$

consequently 0.0206 mmol acetate are required for the production 1.0 mg of cell dry weight (Widdel and Pfennig 1981). The amount of acetate assimilated was subtracted from the total amount of acetate used, to calculate the amount of acetate dissimilated. The average growth yield and standard deviation at each temperature was calculated by standard methods. Standard *t*-tests were performed to verify the significance of differences in growth yield between temperatures.

Fatty acid analysis

Cells for comparison of fatty acid patterns at different temperatures were cultivated in glass bottles containing 1.0 l MV-medium with acetate (20 mM) and sulfate (20 mM), under a N_2:CO_2 (9:1 (v/v)) headspace. The bottles were inoculated with 60 ml of a culture growing exponentially at 15 °C and placed at 4 and 20 °C. The cells were harvested in the exponential phase, by centrifugation for 30 min at 25,000 × *g* and the cell pellet was freeze-dried.

Fatty acid analysis was carried out at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) according to slightly modified standard protocols described in Miller (1982) and Kuykendall et al. (1988). The whole cellular fatty acid pattern is assumed to be essentially the same as the pattern of the cytoplasmic membrane in SRB (Wilkinson 1988; Rütters et al. 2001).

Determination of sulfate reduction rates (SRR)

Sulfate reduction rates were determined in a short-term incubation experiment in a temperature gradient block using the radiotracer method (Jørgensen 1978). Incubations were carried out in duplicates at temperatures ranging from −3.8 to 32.7 °C. The culture was grown in 1.0 l of MV-medium at 15 °C to the exponential phase. Ten millilitres of culture aliquots were transferred aseptically to sterile 15 ml Hungate tubes. The gas phase was flushed with N_2/CO_2 (9:1 (v/v)), and the tubes where sealed with butyl rubber stoppers. Before transfer to Hungate tubes acetate (20 mM) and Na_2SO_4 (20 mM) were added to the stock culture. Inoculated Hungate tubes were preincubated for 2 h in the temperature gradient block to allow temperature calibration. Afterwards $^{35}SO_4^{2-}$ tracer (Risø, Denmark) (50 kBq total radioactivity in 100µl MV-medium) was injected to the culture through the butyl rubber stoppers. The cultures were incubated for 6 h. The incubations were stopped by injection of 1 ml 20% (w/v) zinc acetate through the stoppers and shaken vigorously to precipitate all sulfide present in the headspace. Controls were stopped by injection of 1 ml 20% (w/v) zinc acetate before the addition of the $^{35}SO_4^{2-}$ tracer.

The ^{35}S-labelled sulfide was released from the cultures by a combined acid chromium distillation (Fossing and Jørgensen 1989). Hundred microlitres culture aliquots were withdrawn for determi-

nation of total ^{35}S activity. The remaining liquid was transferred to a N_2-purged distillation apparatus for separation of ^{35}sulfide and ^{35}sulfate. Acid volatile sulfide ($H_2{}^{35}S$ and $Fe^{35}S$) was released with cold 6 N HCL and trapped in 10 ml of a 5% (w/v) zinc acetate solution. After termination of acid distillation the trap was removed and a new was put in place to collect the ^{35}S-sulfide released by the heated chromium acid distillation ($Fe^{35}S_2$ and $^{35}S°$). The samples were analysed and the SRR calculated as described by Fossing and Jørgensen (1989). For direct cell counts an exponentially growing culture was homogenized with an ultrasonic probe (Bandelin, Berlin, Germany) applying the lowest intensity possible (10% of max for 2 times 20 s). Cells were fixed in formalin (8% (v/v)) and counted under the microscope in a Bürker-Türk chamber. The average cell count was used for determination of the cell specific SRR.

Chemical analysis

Sulfate and acetate concentrations were determined by HPLC and/or suppressed ion chromatography on a Sykam (Sykam, Gilching, Germany) or a Dionex system (Dionex, Sunnyvale, CA). Sulfate was separated from the other anions with an anion separation column LCA A14 (Sykam) at 60 °C with Na_2CO_3 (7.5 mM), 50 mg/l 4-hydroxybenzenonitrile in 50 ml 96% v/v ethanol as eluent (flow rate = 1.5 ml min^{-1}). Diluted (20 times in eluent) samples (60 µl) were injected onto the column. Acetate concentrations were determined after separation from other compounds on a Sarasep CAR-H column at 60 °C with H_2SO_4 (5 mM) as eluent (flow rate = 0.9 ml min^{-1}). Sixty microlitres of diluted sample (diluted 20 times in Milli-Q water) was injected onto the column. On the Dionex system sulfate and acetate were measured simultaneously. The components were separated on an IonPac AS18 Analytical (4 × 250 mm) No. 060549 column, with KOH (23 mM) as eluent at 60 °C at a flow adjusted to 1.0 ml min^{-1}. Diluted sample (20 µl, diluted 20 times in Milli-Q water) was injected on the column.

Arrhenius plot and Q_{10}

The effect of temperature on growth or sulfate reduction rates was investigated by application of the Arrhenius equation as previously described by Isaksen and Jørgensen (1996), Knoblauch and Jørgensen (1999) and Rabus et al. (2002):

$$\ln(k) = \ln(A) - (E_a/R \cdot 1/T),$$

where k is the growth or the sulfate reduction rate, A is the Arrhenius constant, R is the gas constant (8.31 J K^{-1} mol^{-1}) and T is the absolute temperature (K). The activation energy E_a describes the response of the specific SRR or growth rate of bacteria to temperature changes (Mohr and Krawiec 1980). The activation energy E_a (J mol^{-1}) was estimated from the slope of the linear part of the Arrhenius plot of ln (SRR) and ln (growth rate) as a function of T^{-1}. Q_{10} was calculated from the following equation:

$$Q_{10} = \exp[(E_a \cdot 10)/(RT(T + 10)]$$

G + C content and DNA–DNA hybridization

The G + C content of genomic DNA was determined at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) according to standard methods described in Cashion et al. (1977), Tamaoka and Komagata (1984), Mesbah et al. (1989). DNA–DNA hybridization between strain akvb^T, *D. hydrogenophilus* and *D.* sp. ASv25 was also performed at the DSMZ according to standard protocols described in Cashion et al. (1977) and Deley et al. (1970) considering the modifications described by Huss et al. (1983).

Nucleic acid extraction, PCR amplification and sequencing

Genomic DNA was extracted using the FastDNA spin kit for soil (Bio101, Vista, California) according to the protocol of the manufacturer. Almost complete 16S-rRNA gene sequences were obtained with the following primers: Eub-26F (5′-agagtttgatcctggctca-3′) (Hicks et al. 1992) and Eub-1392R (5′-acgggcggtgtgtac-3′) (Zheng et al. 1996). The PCR product was purified using Gel Elute^{TM} PCR Clean-up Kit (Sigma-Aldrich, Copenhagen, Denmark) according to the manufacturer's protocol. The purified product was sequenced with an ABI 3100 DNA sequencer

using a DYEnamic ET Terminator Sequencing Kit (Amersham Biosciences, Uppsala, Sweden). In addition to primers Eub-26F and Eub-1392R, primers Eub-518F (5′-ccagcagccgcggtaat-3′), Eub-518R (5′- attaccgcgctgctggg-3′), Eub-341F (5′-cctacgggaggcagcag-3′) (Muyzer et al. 1993) and Univ-907F (5′-aaactyaaaggaattgacgg-3′) modified after Lane (1991) were used for sequencing.

Phylogenetic analysis

Sequences were compiled and aligned using the ARB program package (Ludwig et al. 2004) using the automatic aligning tool. Alignments were subsequently corrected manually where necessary. The closest relatives of strain akvb^T were found by comparing the 16S rRNA gene sequence of akvb^T to the Genbank database using the BLAST algorithm provided by the National Centre for Biotechnology Information (Wheeler et al. 2002). The phylogenetic relationship of the compiled sequences was tested using reference taxa from representatives from all authenticated species of the genus *Desulfobacter* as well as various outgroup taxa. Phylogenetic analysis was performed by distance-matrix (neighbour-joining), maximum-parsimony and maximum-likelihood algorithms available in the ARB programme package. Three different filters were applied to include or exclude sequence positions in the analysis, respectively. The *deltaproteobacteria* filter and the eu-bacterial filter of the ARB database version Jan_2002 (Ludwig et al. 2004) and a filter including unambiguously aligned positions of the 16S-rRNA gene sequence of strain akvb^T were calculated by maximum-parsimony analysis using the ARB database version Jan_2002 (Ludwig et al. 2004).

Results

Enrichment and isolation

The precipitation of FeS in enrichment cultures derived from surface sediments, grown in the iron-rich CMS medium, indicated the presence of sulfate reducing bacteria. Microscopic inspection revealed the presence of cell clumps attached to the precipitate. The clumps were dominated by large thick rods and elongated rods with round ends. After four passages through deep agar dilutions pure cultures were obtained, of which one culture, designated strain akvb^T, was studied in detail. Cells of strain akvb^T were thick rods with round ends. They were 1.5–2.5 μm wide and 3–5 μm long. The cells grew in dense aggregates often attached to FeS precipitates (Figure 1). Old cultures formed larger aggregates, especially when grown at higher temperatures (>10 °C) and expressed variable swollen cell shapes. At lower temperatures (<5 °C) the cells were elongated. Microscopy revealed that the cells were motile.

Growth conditions and nutrition

Growth characteristics of strain akvb^T and other validly described members of the genus *Desulfobacter* are given in Table 1. The temperature range of strain akvb^T was −3.6 to 26.3 °C with an optimal growth temperature (T_{opt}) at 20 °C (see Figure 2a). The maximum growth rate was 0.4 d^{-1} giving a doubling time (t_d) of 40 h, which is twice as long as the shortest doubling time reported for other *Desulfobacter* spp. grown on acetate. The optimal pH for growth was between pH 7.2 and 7.4. Strain akvb^T was routinely grown in the presence of 20 g l^{-1} NaCl. It grew from 3 to 40 g l^{-1} NaCl with an optimum ranging from 16 to 26 g l^{-1} NaCl. Vitamins were not required for growth. Of the substrates tested, strain akvb^T grew exclusively by the oxidation of acetate coupled to the reduction of sulfate (see Table 1).

Sulfate reduction rate measurements

In a short-term sulfate reduction rate experiment, strain akvb^T showed the highest cell specific sulfate reduction rate (6.2 fmol $cell^{-1}$ d^{-1}) at 26 °C (see Figure 3a), which is 6 °C above T_{opt} for growth and near the maximum temperature (T_{max}). The temperature range of sulfate reduction exceeded the temperature range of growth by 5 °C.

Arrhenius relationship of growth and sulfate reduction

The Arrhenius relationship for the temperature dependence of growth (Figure 2b) and sulfate

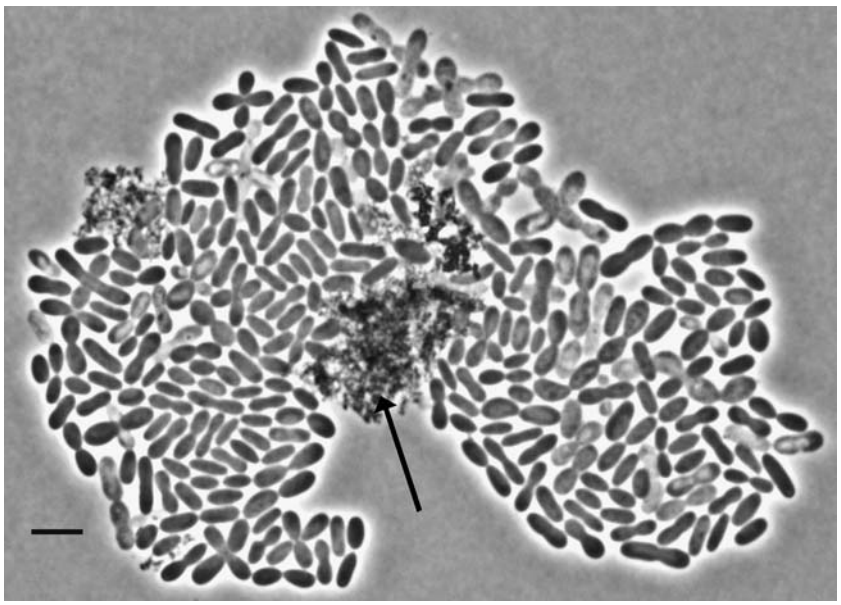

Figure 1. Phase-contrast photomicrograph of strain akvb^T. The reference bar is 5 μm. The arrow points to precipitated ferrous sulfide, around which the cells form aggregates.

reduction (Figure 3b) was linear with two distinct slopes below the optimum temperatures of both processes (20 and 26 °C, respectively). The point of inflection is identified as the 'critical temperature', $T_{critical}$ (Mohr and Krawiec 1980; Guillou and Guespin-Michel 1996; Bakermans and Nealson 2004). $T_{critical}$ for growth and sulfate reduction was 6.4 °C.

The activation energy E_a and Q_{10} for strain akvb^T was calculated from the two slopes of the Arrhenius plots. E_a for the growth rate was 130.2 kJ mol^{-1} for the low (−3.6 to 6.4 °C) and 64.3 kJ mol^{-1} for the high temperature range (6.4 to 20 °C), respectively. Q_{10} was 8.0 in the range from −3.6 to 6.4 °C and 2.6 in the range from 6.4 to 16.4 °C, respectively. E_a for sulfate reduction was 121.9 kJ mol^{-1} for the low (−3.6 to 6.4 °C) and 41.5 kJ mol^{-1} for the high temperature range (6.4 to 23.6 °C), respectively. The corresponding Q_{10} value was 7.0 in the range from −3.6 to 6.4 °C and 1.9 in the range from 6.4 to 16.4 °C, respectively.

Growth yield at different temperatures

Strain akvb^T had the highest growth yield (4.3–4.5 g dry weight mol^{-1} acetate) at temperatures between 5 and 15 °C (Figure 4). At 20 °C, the temperature at which strain akvb^T grew fastest, the growth yield was 3.5 g dry weight mol^{-1} acetate. The growth yield dropped significantly between 5 and 0 °C. *t*-tests confirmed that reduced yields, measured at 20 and 0 °C, were significantly different from the yield measured at 10 and 15 °C ($p < 0.05$). No differences in growth yield were found if cells were grown at 5, 10 and 15 °C ($p > 0.05$).

Fatty acid compositions at different temperatures

The cellular fatty acid compositions of strain akvb^T grown at 20 and 4 °C are summarized in Table 2. The saturated (*n*-14:0) and (*n*-16:0) and the unsaturated (16:1ω7c) fatty acids were dominant at both temperatures. The amounts of unsaturated and saturated fatty acids were almost the same at 20 °C. The sum of unsaturated fatty acids was clearly higher at 4 °C compared to 20 °C, while the sum of saturated fatty acids was 8% lower. A small increase in the amount of short-chained (< 16 C-atoms) fatty acids and a decrease of branched fatty acids was observed between cultures grown at 20 and 4 °C. The relative amount of the quantitatively dominant unsaturated fatty acid (16:1ω7c) was about 10% higher at 4 °C than at 20 °C. Concomitantly, the relative

Table 1. Comparison of characteristics of strain akvb^T and validly described species of the genus *Desulfobacter*.

Characteristics	Strain akvb^T	*D. hydrogenophilus*[a]	*D. vibrioformis*[b]	*D. curvatus*[a]	*D. halotolerans*[c]	*D. latus*[a]	*D. postgatei*[d]
Cell shape	Thick rod, round ends	Oval, elongated	Curved rods	Curved rods	Oval, elongated	Oval, elongated	Oval
Cell sizes (μm)							
Width	1.5–2.5	1–1.3	1.9–2.5	0.5–1	0.8–1.2	1.6–2.4	1–1.5
Length	3–5	2–3	4.5–8	1.7–3.5	3–5	4–7	1.7–2.5
Motility	+	–	+	+	+	±	±
Temp. range/optimum (°C)	−6.0[f]–26.3/20.1	0–35/29–32	5–38/33	/28–31	7–38/32–34	/29–32	/32
pH optimum	7.2–7.4	6.6–7.0	6.8–7.0	6.8–7.2	6.2–7.4	7.0–7.3	7.3
NaCl range/optimum (% wt/vol.)	0.3-4.0/1.6–2.6	≤ 6[c]/1	1–5/≥0.3	/≥0.7	0.5–13/1–2	≤ 6[c]/2	/≥0.5
G+C content of DNA (mol%)	45.2	44.6	47	46.1	49	43.8	45.9
Vitamin requirement(s)	None	Biotin, 4-aminobenzoate	None	Biotin	Biotin, 4-aminobenzoate	Biotin, thiamin	Biotin, 4-aminobenzoate
Electron donors[e]							
Acetate (20)	+	+	+	+	+	+	+
H_2 (autotrophic)	–	+	–	+[h]	–	–	–
Ethanol (10–20)	–	+	–	+	+	–	–
Pyruvate (10)	–	+	–	+	+[h]	–	–
Butyrate (5–10)	–	–	–	–	–[g]	–	–
Electron acceptors[e]							
Sulfate (≤ 30)	+	+	+	+	+	+	+
Sulfite (2–5)	–	+	+	+	+	–	+
Thiosulfate (5–20)	–	+	+	+	+	–	+
Shortest doubling time (h) on acetate	41[i]	18	20	21	22	21	20

[a]Data from Widdel (1987).[b]Data from Lien and Beeder (1997).[c]Data from Brandt and Ingvorsen (1997).[d]Data from Widdel and Pfennig (1981).[e]Added concentrations (in parentheses) are given in mM. The following electron donors were not utilized by strain akvb^T: Propionate (10), lactate (15), succinate (10), malate (10) and H_2+acetate (1). Electron acceptors tested but not utilized by strain akvb^T: Elemental sulfur, nitrate (5), fumarate (10), taurine (0.2), BES (1), Co-enzyme (0.25), ethanosulfonat (1) and ironIIIcitrate (5).[f]From temperature −3.6 to 26.3 °C use of acetate and sulfate was observed.[g]Some H_2S produced but no growth observed.[h]Growth ceased after two transfers.[i]Doubling time determined from the use of acetate.

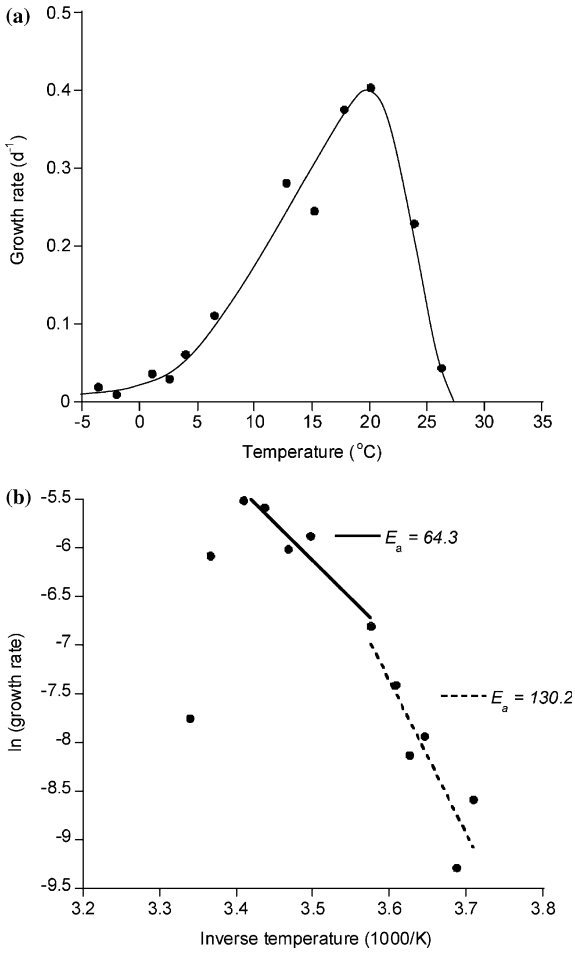

Figure 2. (a) Growth rates of strain akvb^T at different temperatures. Determined from the consumption of acetate during incubations. The points represent an average of two series. The optimal growth rate was at 20 °C. (b) Arrhenius plot of the data in panel (a). E_a values calculated for the two linear slopes on the plot are added (kJ mol^{-1}).

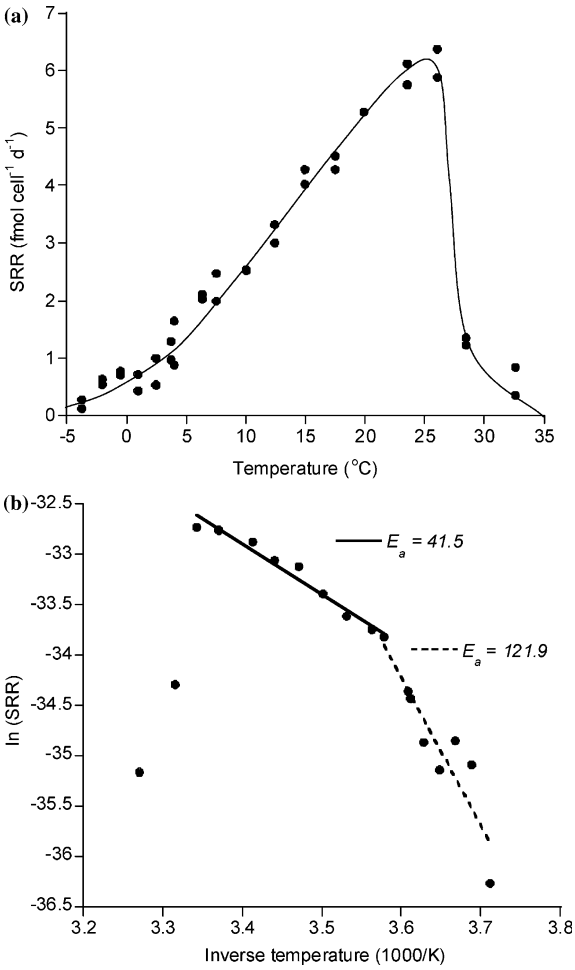

Figure 3. (a) Cell specific sulfate reduction rates of akvb^T at different temperatures. Determined by short-term incubations in a temperature gradient block. The highest activity was at 26 °C. (b) Arrhenius plot of the data in panel (a) represented by the average value of the two series in (a). E_a values calculated for the two linear slopes on the plot are added (kJ mol^{-1}).

amount of the dominant saturated (*n*-16:0) fatty acid decreased by 10%. The quantitatively dominant short-chained fatty acid (*n*-14:0) also increased. The amount of 17:0 cyc was almost the same at the two temperatures.

DNA data and phylogeny

The molar G + C content of DNA from strain akvb^T was 45.2 mol%. The 16S rRNA gene-based phylogenetic analyses clearly placed strain akvb^T within the genus *Desulfobacter* (Figure 5). The 16S rRNA gene sequence of strain akvb^T shared a high degree of similarity with the 16S rRNA gene sequence of *D. hydrogenophilus* (98.6%) and *Desulfobacter* sp. Asv25 (98.3%). The DNA–DNA homology of strain akvb^T and *D. hydrogenophilus* was 32%. The DNA–DNA homology between strain akvb^T and *Desulfobacter* sp. ASv25 was 61.8%.

Discussion

The effect of temperature on growth and sulfate reduction

Based on the seasonal temperature variation at the sample site in the northern part of the North Sea,

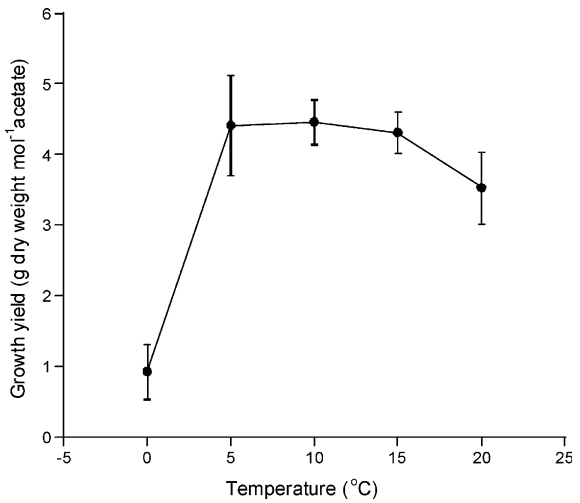

Figure 4. Growth yields of strain akvb^T. Standard deviation for the three series is added. The maximum growth yield was at 5–15 °C.

which ranges from 8 to 14 °C, we expected to isolate psychrotolerant rather than mesophilic sulfate-reducing bacteria. Our isolate, strain akvb^T, was psychrotolerant . Strain akvb^T grew at −3.6 °C with doubling times of about 2 months. This is to our knowledge the lowest growth temperature reported for a sulfate reducer so far. Despite the fact that the minimum temperature (T_{min}) for growth was below the temperature reported for both psychrophilic and psychrotrolerant sulfate-reducing bacteria (Widdel 1987; Isaksen and Teske 1996; Sass et al. 1998; Knoblauch and Jørgensen 1999; Rabus et al. 2002) strain akvb^T is still considered psychrotrolerant, because its T_{opt} is 20 °C and its maximum growth temperature (T_{max}) is between 26 and 28 °C (Morita 1975; Russell 1990). It is commonly observed among psychrotolerant microorganisms and psychrophiles that their T_{opt} is above the *in situ* temperature of the natural environment of the bacteria (Delille and Perret 1989; Isaksen and Jørgensen 1996; Knoblauch and Jørgensen 1999). Here, however, it should be kept in mind that growth experiments are generally carried out at unnaturally high substrate concentrations, thus excluding growth efficiency and K_s as selecting parameters. This contrasts the conditions encountered in the habitat, where growth substrates are usually lim-

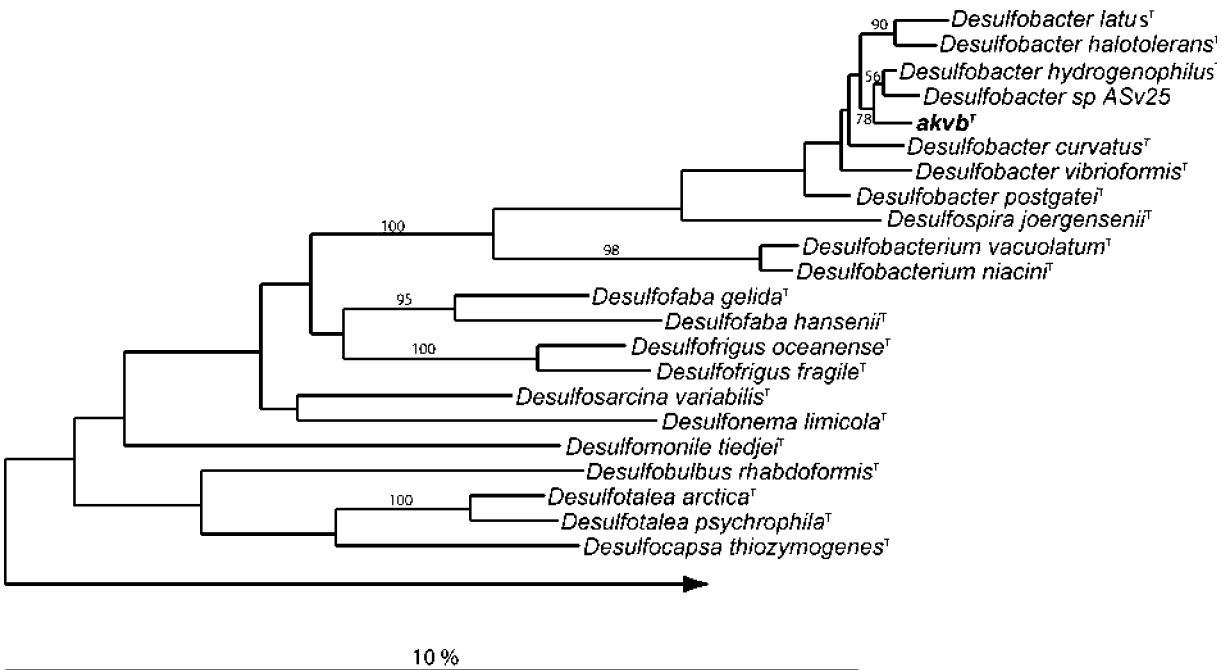

Figure 5. Phylogenetic relationship of the 16S rRNA gene sequence from strain akvb^T and selected reference taxa of the *Delta-proteobacteria* class. The tree was constructed by the Neighbour-joining algorithm using a 50% conservation filter to include or exclude sequence positions in the analysis. Evolutionary distances were calculated with Jukes-Cantor distance correction. Bootstrap values calculated by maximum-parsimony analysis are shown at nodes receiving > 50% bootstrap support. The bar represents 10% estimated sequences divergence.

Table 2. Relative amount of cellular fatty acids (%) of strain akvb^T, incubated at 4 and 20 °C.

Fatty acids	Temperature	
	20 °C	4 °C
n-12:0	0.5	0.5
13:0 *iso*	0.9	0.6
n-14:0	11.8	14.5
14:0 *iso*	0.3	–
14:1ω5c	–	0.4
14:0 3-OH	1.1	1.6
15:0 *iso*	1.9	0.5
15:0 *anteiso*	0.3	–
15:1 *iso*f[d]	0.5	0.5
15:0 *iso* 3-OH	0.9	0.6
n-16:0	28.8	18.4
16:1ω5c	3.1	4.9
16:1ω7c	31.6	42.2
16:1ω9c	1.1	2.0
16:0 3-OH	2.3	2.4
16:0 *iso*	0.3	–
17:0 *iso*	0.8	–
17:1 *iso* ω9c	0.2	–
17:0 cyc	4.3	4.1
n-18:0	1.2	0.6
18:1ω7c	7.8	6.0
18:1ω9c	0.5	–
Unidentified	0	0.2
Unsaturated[a]	48.5	59.6
Saturated[b]	46.4	38.1
Short chain (< 16 c-atoms)	18.2	19.1
Branched[c]	6.0	2.2

Abbreviation exemplified by: 14:0, tetradecanoic acid; 14:1ω5c, 5-tetradecenoic acid, double bond *cis*-standing; 14:0 3-OH, 3-hydroxy-tetradecanoic acid; 15:0 iso/anteiso, pentadecanoic acid, iso-/anteiso-branched; 17:0 cyc, heptadecanoic acid, cyclopropane. [a]Total unsaturated fatty acids including hydroxylated chains. [b]Total saturated fatty acids including straight and cyclopropane. [c]Total branched fatty acids including iso and anteiso. [d]The position of the double bond is unknown.

iting and thus substrate affinity and the efficiency of transforming substrate energy into biomass decide upon survival and proliferation of the microbial cell. Strain akvb^T conserved energy by coupling the oxidation of acetate to the dissimilatory reduction of sulfate. SRR were highest at temperatures from T_{opt} to T_{max} for growth.

Accordingly, sulfate reduction optima which were up to 10 °C higher than the optimum temperature for growth have been found in the other psychrotolerant and psychrophilic sulfate reducers (Isaksen and Jørgensen 1996; Knoblauch and Jørgensen 1999; Rabus et al. 2002). The fact that the SRR peaked at temperatures exceeding T_{opt} and T_{max} for growth indicates that sulfate reduction is partially uncoupled from growth at these temperatures, correlating with a decreasing growth yield. Some of the energy that is conserved by sulfate reduction at high temperatures is possibly used for repair and maintenance rather than for cell division and biosynthesis.

The adaptation of strain akvb^T to different temperatures can be determined from the Arrhenius plots. A plot with a straight line is interpreted as a good temperature adaptation of the culture to temperatures within the temperature interval (Harder and Veldkamp 1968; Sagemann et al. 1998). The lower the activation energy (E_a) and Q_{10}, the less affected are the growth or sulfate reduction rates by decreasing temperatures. Strain akvb^T showed two distinct linear domains separated by the critical temperature ($T_{critical} = 6.4$ °C) for both growth and sulfate reduction. The higher Q_{10} values for growth rate, compared to sulfate reduction rate in both temperature domains, might indicate that the complex series of integrated enzymatic reactions involved in the production of cells is more negatively affected by changes in temperature, than the enzymes involved in the less complex sulfate reduction process. The E_a value calculated for sulfate reduction rates for strain akvb^T from T_{opt} to 6.4 °C (41.5 kJ mol^{-1}) were lower than the values reported for other psychrotolerant sulfate reducers (54–86 kJ mol^{-1}) (Knoblauch and Jørgensen 1999). The low value indicates that cell energy metabolism for strain akvb^T is less affected than growth by decreasing temperatures. This might be an advantage for strain akvb^T since this temperature range is close to the annual *in situ* temperature range of its natural habitat. The two to three fold increases in E_a in both growth rate and sulfate reduction rate below $T_{critical}$ indicates a pronounced decrease by small temperature changes. A similar pattern was described for sulfate reduction in *D. hydrogenophilus* (Bak 1988), which in the temperature range from 0 to 28 °C had a $T_{critical}$ at 8 °C and a 3-fold increase in E_a below $T_{critical}$. For other sulfate-reducing bacteria that were able to grow at 0 °C only continuous linearity in the Arrhenius plot from T_{min} to T_{opt} has been reported (Isaksen and Jørgensen 1996; Knoblauch and Jørgensen 1999; Rabus et al. 2002). These isolates might therefore be better adapted to the cold environments from where they were isolated compared to strain akvb^T, which was isolated from an environment with large tempera-

ture fluctuations and a higher average annual temperature. However, a bimodal pattern might also have been observed in the investigations by Isaksen and Jørgensen (1996), Knoblauch and Jørgensen (1999) and Rabus et al. (2002) if more temperatures in the low temperature range had been included.

The effect of temperature on growth yield

Acetate is one of the most important end products of fermentation pathways and it is one of the major substrates for sulfate reducers in temperate marine environments (Sørensen et al. 1981; Parkes et al. 1989). Strain akvb^T had maximum growth yields when grown on acetate at temperatures from 5 to 15 °C, (between 4.3 and 4.5 g mol^{-1} acetate). These growth yield values were in the same range as values reported for the acetate-oxidizing psychrophilic SRB *Desulfofrigus oceanense* (4.9 g mol^{-1} acetate, determined at 7 °C) and for the much faster growing, mesophilic acetate-oxidizing *Desulfobacter* strains (4.5–4.8 g mol^{-1} acetate) (Widdel and Pfennig 1981; Widdel 1987; Brandt and Ingvorsen 1997; Lien and Beeder 1997). A maximum growth yield at 5–15 °C indicated that strain akvb^T is well adapted to the seasonal range of temperatures from 8 to 14 °C. The two psychrotolerant *Desulfovibrio* species described by Sass et al. (1998) had maximum growth yields below T_{opt}. The growth yield pattern of the psychrotolerant *Desulforhopalus vacuolatus* showed a constant high growth yield from 0 to 10 °C and a lower growth yield near T_{opt} (Isaksen and Jørgensen 1996). The psychrotolerant and psychrophilic sulfate reducers described by Knoblauch et al. (1999): *Desulfofaba gelida*, *D. oceanense*, *Desulfofrigus fragile*, *Desulfotalea artica* and *Desulfotalea psychrophila*, had either an almost constant growth yield between 0 °C and T_{opt} or expressed the highest growth yields at temperatures around 0 °C (Knoblauch and Jørgensen 1999). Thus it seems that the above mentioned cold adapted sulfate-reducing bacteria including strain akvb^T have higher growth yields at temperatures below T_{opt} and closer to the *in situ* temperature than to T_{opt}. These observations support the above-presented argument that growth yields rather than growth rates should be considered when the temperature adaptation of a microbe is evaluated.

The ability to maintain a high growth rate, energy metabolism and growth yield at *in situ* temperatures indicates that strain akvb^T is well adapted to the temperature regime of the environment from which it was isolated. Strain akvb^T might therefore have competitive advantages over mesophilic or psychrophilic sulfate-reducing bacteria, which have more narrow temperature spectra. Our isolate may thus play an important role in the anaerobic degradation of acetate in this region of the North Sea.

The effect of temperature on the cellular fatty acids composition

Maintenance of membrane fluidity at low temperature is vital for membrane and consequently cell functionality. At lower temperatures the membrane tends to solidify, but an increase in the relative amount of unsaturated fatty acids leads to an increase in fluidity. A change in the ratio of saturated to unsaturated fatty acids is the most common observed regulation of membrane fluidity in bacteria (reviewed in Russell (1990), Russell and Hamamoto (1998) and Denich et al. (2003)).

In psychrophilic SRB investigated so far, constant high levels of unsaturated fatty acids (around or above 80%) were found (Könneke and Widdel 2003). These are the highest levels found in SRB at low temperatures and may be an adaptation to permanently cold environments (Knoblauch et al. 1999; Könneke and Widdel 2003). Other SRB, including several mesophilic *Desulfobacter* species (Könneke and Widdel 2003) and the psychrotolerant *Desulfobacterium autotrophicum* (Rabus et al. 2002), increased the proportion of unsaturated fatty acids with decreasing temperature. This pattern was also observed in strain akvb^T when cultures were grown at 20 or 4 °C. Generally, *Desulfobacter* species seem to have a rather sensitive system, that regulates the fatty acid composition as a function of temperature changes (Könneke and Widdel 2003). It has been speculated that the ability to change and regulate the fatty acid composition of the cell, might be an adaptation to environments with fluctuating temperature regimes (Könneke and Widdel 2003). *D. hydrogenophilus*, the phylogenetically closest relative of strain akvb^T, increased the relative amount of unsaturated fatty

acids from 30 to 43 to 65% when grown at 28, 20 or 4 °C, respectively (Könneke and Widdel 2003), while the relative amount of unsaturated fatty acids of strain akvb^T increased from 48 to 59% when grown at 20 and 4 °C (Tables 2 and 3). Cellular fatty acids have previously been suggested as biomarkers, which could indicate the presence of distinct genera of SRB in the natural environments (Taylor and Parkes 1985; Dowling et al. 1986). However, the reliability of the fatty acid 16:0 10Me as a biomarker for *Desulfobacter* species has been questioned, as this fatty acid has also been found in other genera of SRB (Kohring et al. 1994; Kuever et al. 2001; Rabus et al. 2002) and only at very low levels in *D. hydrogenophilus* when grown at low temperatures (Table 3; Könneke and Widdel 2003). The absence of this marker fatty acid in strain akvb^T, at both temperatures (Table 3), highlights the limitations of using fatty acids as genus specific biomarkers.

Taxonomic affiliation

Based on 16S rRNA gene sequence analysis strain akvb^T can be affiliated to the genus *Desulfobacter*. Strain akvb^T shares with the other members of the genus the ability to oxidize acetate completely to CO_2, with sulfate as electron acceptor (Widdel and Pfennig 1981; Widdel 1987; Brandt and Ingvorsen 1997; Lien and Beeder 1997). Phylogenetically, the closest validly described relative of strain akvb^T is *D. hydrogenophilus* sharing a 16S rRNA gene sequence similarity of 98.6%. The DNA–DNA hybridization value of about 32% clearly separates the two strains into two distinct species (Wayne 1987). In addition the two strains can be easily distinguished phenotypically (Table 1). Most importantly, strain akvb^T exclusively used sulfate as electron acceptor and acetate as electron donor and carbon source, whereas *D. hydrogenophilus* has a more versatile substrate spectrum. In addition, *D. hydrogenophilus* required vitamins for stable growth, which was not the case for strain akvb^T. Furthermore, *D. hydrogenophilus* has lower NaCl and pH optima than strain akvb^T (Table 1). Although *D. hydrogenophilus* is described as psychrotolerant with the ability to grow at 0 °C it has a much higher temperature optimum for growth (29–32 °C) than strain akvb^T (Table 1). The cellular fatty acid composition is also different between the two strains, with 10Me-16:0 being absent from strain akvb^T.

Based on the above-mentioned phenotypic and DNA-based characteristics we consider strain akvb^T to be a new species, for which we propose the name *Desulfobacter psychrotolerans* sp. nov.

Description of Desulfobacter psychrotolerans *sp. nov.*

Desulfobacter psychrotolerans (psy.chro.to'le.rans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. neut. part. adj. *psychrotolerans* tolerating cold temperatures).

Cells are motile, rod shaped with round ends, 1–3 μm wide and 2–5 μm long. Cells expressed pleomorphic morphologies in old culture. Cells grow as aggregates, which often attach to precipitates in the growth medium. Sulfate is reduced with acetate as the sole electron donor and carbon source. No vitamins are required for growth.

Table 3. Comparison of the fatty acid composition of *D. hydrogenophilus* and strain akvb^T at two different temperatures.

Fatty acids	*D. hydrogenophilus*		Strain akvb^T	
	[b]28/[a]30 °C (%)	[b]12°C (%)	[c]20°C (%)	[c]4 °C (%)
n14:0	n.d./0	n.d.	11.8	14.5
15:0 iso	n.d./13	n.d.	0.5	0.5
n16:0	30/30	30	28.8	18.4
16:1ω7c	15/1.3	45	31.5	42.2
10Me-16:0	5/4.3	1	0	0
17:0cyc	15/39	5	4.2	4

n.d. – not determined. [a]Kohring et al. (1994). [b]Könneke and Widdel (2003).[c]This study.

Temperature range for growth is from −3.6 to 26.3 °C with an optimum at 20.1 °C. pH optimum for growth is pH 7.2–7.4. No growth occurs below pH 6.1 or above pH 8.0. Growth occurs in the presence of 0.3–4% w/v NaCl with an optimum between 1.6 and 2.6% w/v NaCl. Major cellular fatty acids are *n*-14:0, *n*-16:0 and 16:1ω7c. 10Me-16:0 is absent. The molar G+C content is 45.2 mol%. The type strain akvb^T has been isolated from marine surface sediment of a methane seep (the North Sea) (=DSMZ 17155=ATCC BAA-1150=GenBank DQ057079).

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