Isolation and Characterization of Psychrophilic Sulfate-Reducing Bacteria from Permanently Cold Arctic Sediments

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PREFACE

The present work is part of an ongoing research project on the regulation of organic matter decomposition in the Arctic ocean. In this project special attention is paid to anaerobic mineralization processes such as sulfate reduction. The work was conducted at the Max Planck Institute for Marine Microbiology in Bremen, financed by the Max Planck Society, Munich and is presented as a Ph. D. thesis at the University of Bremen.

The background of the presented study were findings of high sulfate reduction rates in polar sediments and the lack of cultivated sulfate reducers that are able to grow at sub-zero temperatures. The main aim was to isolate and investigate sulfate reducers that are adapted to permanently cold habitats. The isolates obtained were characterized physiologically and phylogenetically to establish whether they are closely related to or completely different from known sulfate reducers. Finally, the adaptation of the new isolates to permanently low temperatures was studied in different pure culture experiments.

The research project on low-temperature adapted sulfate reducers at the Max Planck Institute, Bremen was initiated by Prof. Dr. Bo Barker Jørgensen, the supervisor of the presented work, and I want to thank him first of all for his interest and many inspiring discussions. He is co-author on all manuscripts since he attended the studies with helpful advice and contributed to the work with valuable ideas. Prof. Dr. Friedrich Widdel is acknowledged for many helpful discussions and Prof. Dr. Horst Schulz for his work as a referee of this thesis. Prof. Dr. Donald E. Canfield is thanked for the organization of the Svalbard cruise in fall 1995 during which all samples for the following investigations were taken. I am grateful to Dr. Kerstin Sahm who did the phylogenetic studies on the new isolates, for an inspiring collaboration, for her help during the characterization of the various isolated cultures, and for

supporting words at times when not everything went as it should. Dr. Jens Harder is thanked for his introduction to anaerobic cultivation techniques and his help during the plannings of the Svalbard cruise 1995. I am indebted to Bettina Strotmann for a nice time in our shared office. Dr. Timothy Ferdelman and Dr. Carsten Schubert are thanked for many inspiring discussions and their open ear and patience to all questions about english orthography and grammar. I am also indebted to Jakob Zopfi for his help during the preparation of the manuscripts, for many inspiring discussions and for his support at difficult times of the work. I want to thank Dr. Volker Brüchert and Dr. Carol Arnosti who critically read different versions of the manuscripts and their help in improving the English. Dr. Jens Sagemann is thanked for constructing the temperature gradient block and for many interesting discussions. Swantje Fleischer and Kisten Neumann are thanked for their excellent help with the cultivation of the new isolates and chemical analysis, and Volker Meyer, Gerhardt Kothe, Georg Herz and Olaf Eckhoff for constructing the equipment necessary for this work.

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1. GENERAL INTRODUCTION

Sulfur is an essential element of all living matter and its biogeochemical cycle is closely coupled to that of carbon. Approximately 4% of the Earth's sulfur is present in the hydrosphere, mainly in the form of dissolved sulfate in seawater, whereas about 96% is present as solid sulfur in the pedosphere, a large fraction as sulfide minerals such as pyrite (Holser *et al.*, 1988). Weathering of sulfide minerals in the presence of oxygen is accompanied by oxidation and concomitant production of sulfuric acid. Oxidation can both occur as a purely chemical process but most often it is mediated by sulfide-oxidizing microorganisms such as *Thiobacillus* strains that are adapted to low pH values in their environment (Nordstrom and Stoutham, 1997). Sulfate ions, produced from sulfide oxidation or from weathering of sulfate-containing rocks such as gypsum leach through groundwater and are carried by rivers into the ocean, sustaining the sulfate pool in seawater.

Most marine organisms use sulfate as a sulfur source. They reduce it intracellularly to sulfide and incorporate it into their biomass, i.e. into amino acids and enzymes (assimilatory sulfate reduction). However, most sulfate in the oceans is reduced to sulfide by dissimilatory sulfate-reducing bacteria that live in anoxic environments and use sulfate as the terminal electron acceptor to oxidize organic matter (Goldhaber and Kaplan, 1974). Since the present ocean has only few anoxic basins such as the Black Sea or the Cariaco trench, most of the dissimilatory sulfate reduction takes place in anoxic sediment layers. The produced sulfide either becomes oxidized at the interface between reduced sediment layers and the oxidized sediment surface (Jørgensen, 1987), or it precipitates and is buried, mostly as pyrite (Jørgensen *et al.*, 1990).

1.1 Microbial mineralization of organic matter in marine sediments

Most of the organic carbon mineralized in marine sediments is derived from the primary production in the photic zone (Jørgensen, 1983). Organic particles in the water column are degraded as they sink to the sea floor. Consequently the percentage of primary production that is deposited on the sediment surface is a function of ocean depth (Suess, 1980). In shallow shelf areas (0-200 m water depth) 10-50% of the primary production reaches the sediment surface and fuels the high rates of benthic respiration, whereas in pelagic oceans organic matter is predominantly degraded in the water column and respiration rates in deep sea sediments are extremely low (Jørgensen, 1983; Canfield, 1991). During the microbial oxidation of organic matter electrons are transferred from reduced carbon compounds via electron transport chains to oxidized electron acceptors. The most important electron acceptor in the open ocean is oxygen, which is reduced by aerobic organisms to H_2O. However, in shelf sediments oxygen is available only in the uppermost few millimeter, depending on the respiration rates of aerobic organisms and the supply of oxygen by diffusion and advection (e.g. Revsbech *et al.*, 1980). Below the oxygen respiration zone subsequently nitrate, manganese(IV), and iron(III) are reduced, followed by sulfate reduction and finally methanogenesis (Jørgensen, 1983; Lovley, 1991). Although this sequence of preferentially used electron acceptors is generally

Table 1 : Standard free energy changes for oxidation pathways of organic matter in marine sediments. Data from Berner (1980) and Canfield (1993). Electron acceptors are given in bold.

reaction	ΔG^0(kJ mol^{-1}) of CH_2O
$CH_2O + \mathbf{O_2} \rightarrow CO_2 + H_2O$	-475
$5CH_2O + 4\mathbf{NO_3^-} \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-448
$CH_2O + 3CO_2 + H_2O + 2\mathbf{MnO_2} \rightarrow 2Mn^{2+} + 4HCO_3^-$	-349
$CH_2O + 7CO_2 + 4\mathbf{Fe(OH)_3} \rightarrow 4Fe^{2+} + 8HCO_3^- + 3H_2O$	-114
$2CH_2O + \mathbf{SO_4^{2-}} \rightarrow H_2S + 2HCO_3^-$	-77
$2CH_2O + H_2O \rightarrow 2CO_2 + 4H_2$ and $4H_2 + \mathbf{CO_2} \rightarrow CH_4 + 4H_2O$	-58

found in marine sediments, there is not a strict vertical separation between the zones of different respiration processes (Canfield, 1993). The most common explanation for this redox zonation has been the energy yield of the different reactions (Table 1) whereby the terminal processes proceed from the highest to the lowest energy yield (Berner, 1981). However, since nitrate, Fe(III) and Mn(IV) concentrations are low in comparison to sulfate concentrations (28 mM), dissimilatory sulfate reduction is generally the most important anaerobic mineralization process in marine sediments.

In coastal sediments sulfate reduction contributes up to 50% to the total benthic mineralization (Aller and Yingst, 1980; Jørgensen, 1982; Nedwell *et al.*, 1993), but in areas with very high primary productivity and high burial rates of organic matter, sulfate reducers can be responsible for nearly all mineralization of sedimentary carbon (Thamdrup and Canfield, 1996).

1.2 Substrates of sulfate-reducing bacteria

Organic matter degradation in the absence of oxygen requires several types of bacteria that act together and form an "anaerobic food web" (Blackburn, 1987). The end product of one group of organisms serves as the carbon source for another group until most organic matter is mineralized to CO_2. By extracellular enzymatic hydrolysis fermenting bacteria degrade organic polymers such as proteins, nucleic acids and polysaccharides and release low molecular carbon compounds to the environment. For example chitin, which has been estimated to be the second most abundant polysaccharide on earth after cellulose (Tracey, 1957), is degraded by marine chitin fermenters to CO_2, NH_4^+, acetate, formate, ethanol and hydrogen (Pel and Gottschal, 1986). These and other fermentation end products are subsequently the substrates of sulfate-reducing bacteria.

From the first isolation of a sulfate-reducing bacterium (Beijerinck, 1895) and until the late seventies of this century, sulfate reducers were known only to oxidize simple carbon compounds such as lactate, malate, pyruvate or alcohols

incompletely to acetate. These types of organisms were classified into two different genera, *Desulfovibrio* sp. (Postgate and Campbell, 1966) and *Desulfotomaculum* sp. (Campbell and Postgate, 1965). Many attempts to isolate sulfate reducers that oxidize acetate completely to CO_2 failed, and the possibility of complete carbon oxidation was questioned (Postgate, 1959) until the late 1970's when Widdel and Pfennig (1977) isolated a spore-forming sulfate reducer from hog farm waste that could grow on acetate as the sole carbon and energy source. Later, a marine sulfate reducer that grew exclusively on acetate (Widdel and Pfennig, 1981) was isolated as well and given the name *Desulfobacter postgatei*.

The true nutritional diversity of sulfate reducers gradually became clear as more and more pure cultures were described that could oxidize a wide variety of carbon compounds such as fatty acids (chain length C_1-C_{18} [Widdel, 1980]), amino acids (Stams *et al.*, 1985), aromatic compounds (Widdel, 1980; Imhoff-Stuckle and Pfennig, 1983; Schnell *et al.*, 1989), and aliphatic hydrocarbons (Aeckersberg *et al.*, 1991). From the current perspective, sulfate-reducing bacteria are one of the most versatile groups of anaerobic bacteria, both with respect to their carbon sources and their electron acceptors.

However, despite this wide potential substrate spectrum, field studies indicate that fatty acids are the most important fermentation end products in marine sediments with acetate being quantitatively the most significant (Balba and Nedwell, 1982; Mountfort and Asher, 1981; Sansone and Martens, 1982; Christensen, 1984; Parkes *et al.*, 1989; Sørensen *et al.*, 1981; Skyring, 1988). Other low molecular carbon compounds such as propionate and butyrate (Christensen, 1984; Sørensen *et al.*, 1981), lactate, amino acids (Parkes *et al.*, 1989), as well as hydrogen (Sørensen *et al.*, 1981) were also shown to be important electron donors during sulfate reduction under natural conditions.

1.3 Temperature adaptation of sulfate-reducing bacteria

Temperature is one of the most important environmental parameters for microorganisms because it has an impact on all reactions in living cells. The temperature adaptation of microorganisms can first of all be described by their temperature range of growth. Different organisms are assigned to certain groups according to their cardinal temperatures (Morita, 1975; van de Vossenberg *et al.*, 1998; Blöchl *et al.*, 1995), i.e. the lowest temperature where growth occurs (T_{min}, or minimum temperature), the temperature where growth is fastest (T_{opt}, or optimum temperature) and the highest temperature were growth occurs (T_{max}, or maximum temperature). The following definitions of the different groups will be used throughout this study:

obligate psychrophiles	$T_{min} < 0°C$, $T_{opt} < 15°C$, $T_{max} < 20°C$
moderate psychrophiles	$T_{min} \leq 5°C$, $T_{opt} > 15°C$, $T_{max} > 20°C$
mesophiles	T_{opt} 25-40°C, T_{max} 40-45°C
thermophiles	T_{opt} 55-70°C , T_{max} approx. 75°C
hyperthermophiles	T_{opt} 80-110°C

It should be noted, that even though these definitions refer to growth rates, they are also often used to characterize metabolical activity (e.g., oxygen respiration [Thamdrup and Fleischer, 1998], sulfate reduction [Sagemann *et al.*, 1998], or polysaccharide hydrolysis [Arnosti *et al.*, 1998]). However, metabolical activity and growth rate do not necessarily have the same temperature optimum (e.g. Christian and Wiebe, 1974; Isaksen and Jørgensen, 1996).

The temperatures encountered by sulfate reducers in marine sediments range from below 0°C in polar oceans to ≥ 100°C in the geothermally heated sea floor. In sediments at the hydrothermal vent system of the Guaymas basin temperature optima of sulfate reduction were measured at approximately 40, 80, and 103-106°C (Jørgensen *et al.*, 1992). *Archaeoglobus profundus*, a sulfate

reducer isolated near black smokers of the Guaymas basin, has a temperature optimum of 80°C but does not grow above 90°C (Burggraf *et al.*, 1990). The highest growth temperature of any known sulfate reducer was found at 95°C for the sulfate-reducing archaebacterium *Archaeoglobus fulgidus* isolated from shallow-water hydrothermal vents (Stetter, 1988). Results from measurements of sulfate reduction rates and pure culture studies indicate that hyperthermophilic sulfate reducers such as *Archaeoglobus* spp. have temperature optima that are close to their environmental temperatures. However, such high temperatures are rare in marine sediments and these environments can be regarded as extreme.

Shallow sediments of temperate regions are subject to seasonal temperature variations and sulfate reduction occurs between approximately 0 and 30°C (e.g. Jørgensen, 1977; Nedwell and Abram, 1978; Abdollahi and Nedwell, 1979; Westrich and Berner, 1988; Jørgensen, 1996; Arnosti *et al.*, 1998). Most marine sulfate reducers known so far were isolated from such environments. However, more than 90% of marine sediments have an annual mean temperature colder than 4°C and only 2% are warmer than 15°C (Levitus and Boyer, 1994). Consequently, most benthic bacteria must live and grow at low temperatures of < 4°C which must therefore be considered normal rather than extreme.

The temperature range encountered in most marine sediments is in sharp contrast to the temperature range at which sulfate reducers so far isolated are able to grow. With the exception of the thermophilic and hyperthermophilic sulfate reducers (Widdel and Hansen, 1992) and two other strains (see below), all known sulfate reducers are mesophiles, and cannot grow below approximately 15°C (Widdel and Bak, 1992). Since the known sulfate reducers do not grow at temperatures characteristic for most marine sediments, they also cannot be dominant among the active sulfate-reducing communities of these environments. Even though the importance of sulfate reduction for carbon mineralization has been demonstrated in numerous studies, and even though sulfate reducers have now been isolated that oxidize almost all electron donors

shown to be used for sulfate reduction in nature, most of the organisms active at normal marine *in situ* conditions are still unknown. Only two sulfate reducers, *Desulfobacter hydrogenophilus* (Widdel, 1987) and "*Desulforhopalus vacuolatus*" (Isaksen and Teske, 1996) grow between 0 and 20°C and hence might be active at temperatures common in marine sediments.

The temperature response of sulfate reduction was studied in temperate (Abdollahi and Nedwell, 1979; Isaksen and Jørgensen, 1996; Arnosti *et al.*, 1998) as well as in permanently cold marine sediments (Nedwell, 1989; Sagemann *et al.*, 1998; Isaksen and Jørgensen, 1996). All studies reported highest sulfate reduction rates above the *in situ* temperature, but there seems to be a trend of decreasing optimum temperatures with decreasing environmental temperature. Highest sulfate reduction rates were found at 34°C in a tidal flat with seasonal temperatures of 0-30°C (Arnosti *et al.*, 1998) whereas in permanently cold Antarctic sediments (*in situ* temperatures -1.8-1.0°C) the optimum of sulfate reduction was between 18 and 21°C (Nedwell, 1989; Isaksen and Jørgensen, 1996). Furthermore, sulfate reduction rates at low temperatures (0-5°C) in comparison to those at optimum temperatures were always relatively higher in polar than in temperate sediments (Arnosti *et al.*, 1998; Isaksen and Jørgensen, 1996). This indicates that temperate sediments are inhabited by predominantly mesophilic organisms with low respiration rates at low temperatures, whereas polar sediments harbor a community that is adapted to the low environmental temperatures by relatively high metabolical rates in the cold. The latter conclusion is in accordance with the observation of similar sulfate reduction rates in polar and temperate environments (Jørgensen *et al.*, 1990; Nedwell *et al.*, 1993; Thamdrup *et al.*, 1994; Sagemann *et al.*, 1998) which further indicates the presence of sulfate reducers specially adapted to permanently low temperatures. Different authors concluded that carbon mineralization and sulfate reduction in polar oceans are more likely limited by

the availability of organic matter than inhibited by low temperatures (Thingstad and Martinhussen, 1991; Arnosti *et al.*, 1998; Nedwell *et al.*, 1993).

However, nearly all studies on the temperature adaptation of sulfate reduction are based on measurements of metabolical rates rather than bacterial growth, since growth of sulfate-reducing bacteria is difficult to measure in environmental samples (Gilmour *et al.*, 1990; Winding, 1992). A considerable difference between the optimum temperatures of respiration and growth was found for both aerobic and anaerobic bacteria (e.g. Christian and Wiebe, 1974; Isaksen and Jørgensen, 1996) suggesting that measurements of metabolical rates cannot be used to describe the growth potential of low-temperature adapted bacteria.

Pure culture studies of the temperature adaptation of sulfate reducers are scarce. Isaksen and Jørgensen (1996) investigated a mesophilic (T_{opt}=33°C) and a moderately psychrophilic (T_{opt}=18°C) sulfate-reducing bacterium that were both isolated from a temperate estuary with seasonal *in situ* temperatures of 0-20°C. Even though the mesophile respired between 0 and 40°C, growth was restricted to 8-37°C. The moderate psychrophile had also a wider temperature range for respiration (-3-37°C) than for growth (0-23°C). Furthermore, the temperature optimum of sulfate reduction of the psychrophile (28°C) was 10°C above the optimum growth temperature (18°C), whereas by the mesophile both cardinal temperatures were almost the same (35 and 33°C, respectively). Apart from the different optimum growth temperatures of both strains a more remarkable difference was the temperature response of growth yield, which is the amount of carbon substrate oxidized per amount of cell-biomass produced. In the mesophile growth yield was highest close to T_{opt} and decreased below that temperature to very low values close to T_{min}. In contrast, the moderate psychrophile showed a constant growth yield between 0 and 10°C, and a decrease above that temperature. These results indicate that the mesophile would need more carbon substrate to maintain a given population size when

temperature decreases, whereas the psychrophile would need the lowest amount between 0 and 10°C, i.e. at low temperatures. A similar temperature response to that of the mesophilic strain of Isaksen and Jørgensen (1996) was also found in mesophilic sulfate-reducing bacteria isolated from a freshwater sediment (Sass *et al.*, 1998). Their strains grew at temperatures of 4-35°C with a T_{opt} at 28°C, but growth yields were lowest at the lowest temperature, 4°C. These few results indicate that growth yield responds differently in mesophiles and psychrophiles: it stays constant in psychrophiles but decreases in mesophiles when temperature decreases.

The preceding discussion indicates that, when sulfate reduction rates in polar sediments can be comparable to those of temperate sediments (Nedwell *et al.*, 1993; Sagemann *et al.*, 1998) it must require a community of sulfate reducers that is adapted to low temperatures. Hence, polar habitats should serve as convenient sources for the enrichment and isolation of low-temperature adapted organisms. The first sulfate reducer from a permanently cold environment was isolated by Barghoorn and Nichols (1961) from sulfidic sediments of Antarctica. Unfortunately, the growth range of this new strain was not determined and it is not available in culture collections. Another sulfate reducer, *Desulfotomaculum antarcticum* (Iizuka *et al.*, 1969), was also isolated from an Antarctic sediment but this strain could not grow below 10°C which indicates that it might have been present in the sediment in the form of inactive spores and not as an active organism. Also Vainshtein *et al.* (1995) concluded, that the *Desulfotomaculum orientis* strain, which they isolated from permafrost (in situ temperature <-5°C), was not actually active but survived in the 20-30 thousand year old deposits in the form of inactive spores. Sulfate-reducing bacteria growing at temperatures below 0°C, which are characteristic for polar environments, were not isolated at the onset of the presented thesis work and hence could not be studied.

1.3 Aim of the present study

Our understanding of carbon mineralization processes in anaerobic sediments is to a large extent based on studies with pure cultures of relevant organisms. To better understand the regulation of sulfate reduction in cold sediments, also pure cultures active at *in situ* conditions are needed. Hence, a primary aim of the present study was to isolate the most abundant low-temperature adapted sulfate reducers from permanently cold sediments. Secondly the abundance of the isolated strains in the investigated sediments should be quantified. To relate results from pure culture experiments to processes in the natural environment it is important to know to what extent the investigated strains represent the natural community.

Since it was unclear, whether the sulfate reducers active at low temperatures are closely related to known mesophilic organisms or comprise completely new groups, a detailed characterization of the physiology and phylogeny of the obtained isolates was necessary. The most abundant representatives of the sulfate-reducing community in the sediments were thus chosen for further pure culture experiments. These studies should show how and to what extent psychrophilic sulfate-reducing bacteria are adapted to their permanently cold environment.

1.4 Sampling sites

To study the adaptation of sulfate reducers to low temperatures we collected samples from two permanently cold sediments along the coast of Spitsbergen, Svalbard, Arctic ocean (approx. 77°N, 15°E) during a cruise with the RV "Jan Mayen". One sampling site at the west coast of Spitsbergen (Hornsund) had a bottom water temperature of 2.6°C at the time of sampling (September), whereas the temperature of the other site, Storfjord, was -1.7°C which is near the freezing point of seawater. Both sites were situated on the continental shelf at water depths of 155 m and 175 m respectively.

1.5 Overview of the publications

Sulfate reducers, that are able to grow at the low temperatures of polar oceans were isolated in the first study. Reports of similar sulfate reduction rates in polar and temperate sediments raised questions concerning the community size of sulfate reducers in both habitats. Similar respiration rates can be explained either by, A) more organisms in polar environments that respire with lower rates than in temperate environments, or B) similar community sizes in both habitats but with relatively higher respiration rates of the polar population in spite of the low temperature. To test both possibilities we counted sulfate reducers in sediments of Svalbard with the cultivation dependent most probable number method (MPN). The most abundant cultivable sulfate reducers were isolated at temperatures of 0, 4 and 10°C, cell specific respiration rates were measured at the *in situ* temperature of the sampling sites, and compared to those of mesophiles measured at 4-13°C. The results are presented in the following manuscript:

(1) Christian Knoblauch, Bo Barker Jørgensen and Jens Harder: **Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in Arctic marine sediments**

The second author, Bo Barker Jørgensen, initiated the work with psychrophilic sulfate reducers; he contributed to this study with frequent discussions about the methodology and the interpretation of data. He also measured the sulfate reduction rates in sediment cores. Jens Harder introduced me to anaerobic cultivation techniques. He also participated in the planning of the enrichment and isolation of psychrophilic sulfate reducers.
The manuscript is in press in "*Applied and Environmental Microbiology*"

In the second study, the sulfate-reducing community in the studied sediments was characterized by the cultivation-independent methods of quantitative slot-blot hybridization and denaturing gradient gel electrophoresis (DGGE). In contrast to the MPN-method used in the first study, these methods should give a more realistic picture of the distribution of known sulfate reducers. The results of this study are presented in the manuscript:

(2) Kerstin Sahm, Christian Knoblauch and Rudolf I. Amman: **Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine Arctic sediments**

Most of the work in this study was done by Kerstin Sahm. I isolated and cultivated the investigated pure cultures, and did also all studies on the physiology of the presented strains. Rudolf I. Amman contributed with valuable discussions about the interpretation of the results.
The manuscript is in press in "*Applied and Environmental Microbiology*"

While the first two studies focused on the community of sulfate reducers in the investigated Arctic sediments, the third study describes the physiology and phylogeny of five new psychrophilic sulfate-reducing bacteria in detail. The results of this study are presented in the manuscript:

(3) Christian Knoblauch, Kerstin Sahm and Bo Barker Jørgensen: **Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov.**

A substantial contribution to this study was made by Kerstin Sahm who performed all phylogenetic studies. Bo Barker Jørgensen participated in the study with frequent discussions during the isolation of the new strains and supplied many helpful ideas.
The manuscript is in press in the "*International Journal of Systematic Bacteriology*"

The fourth manuscript deals with the temperature adaptation of the five strains described in the third manuscript. These investigations were the first studies of psychrophilic sulfate-reducing bacteria from polar environments and suggest that the psychrophilic isolates have a competitive advantage at low temperature, a feature that distinguishes them from mesophiles. The results of this study are presented in the manuscript :

(4) Christian Knoblauch and Bo Barker Jørgensen: **Effect of temperature on sulfate reduction, growth rate, and growth yield in five psychrophilic sulfate-reducing bacteria from Arctic sediments**

The second author, Bo Barker Jørgensen initiated this study. He contributed many helpful ideas and provided help with the interpretation of data.
The manuscript is in press in "*Environmental Microbiology*".

2. SUBMITTED MANUSCRIPTS

2.1.

Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in Arctic marine sediments

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Numbers of sulfate reducers were determined in two Arctic sediments with *in situ* temperatures of 2.6 and -1.7 °C. Most probable number counts at 10 °C were higher than at 20 °C, indicating the predominance of a psychrophilic community. Mean specific sulfate reduction rates of 19 isolated psychrophiles were compared to corresponding rates of 9 marine, mesophilic sulfate-reducing bacteria. The results indicate that, as a physiological adaptation to the permanently cold Arctic environment, psychrophilic sulfate reducers have considerably higher specific metabolic rates than their mesophilic counterparts at similarly low temperatures.

Dissimilatory sulfate reduction is the most important bacterial process in anoxic marine sediments, accounting for up to half of the total organic carbon remineralization (Jørgensen, 1982; Canfield *et al.*, 1993; Nedwell *et al.*, 1993). Since more than 90% of the global sea floor is cold (< 4°C [Levitus and Boyer, 1994]), sulfate reducers must be able to metabolize and grow at low ambient temperatures. Sulfate reduction rates (SRR) in polar sediments may be similar to those of temperate environments (Nedwell *et al.*, 1993; Jørgensen *et al.*, 1990; Thamdrup *et al.*, 1994; Sagemann *et al.*, 1998), but sulfate reducers active in polar sediments had not been isolated and studied.

Similar sulfate reduction rates in cold and in temperate sediments could be explained either by A) more sulfate reducers in cold environments to compensate for lower per-cell sulfate reduction rates (i.e. cell-specific SRR) at low temperatures, or B) comparable community sizes in both environments but higher specific respiration rates of psychrophiles relative to mesophiles at low temperatures. In the present study, both possibilities were investigated by quantifying sulfate reducers in two polar sediments as well as by comparing specific SRR of new psychrophilic isolates to those of known mesophilic sulfate reducing bacteria (SRB). Because the phylogeny and physiology of sulfate reducers living in polar sediments were previously unknown, we used the most probable number (MPN) method to count and subsequently isolate the most abundant cultivable sulfate reducers for further pure culture studies.

Two permanently cold sediments at the coast of Svalbard, Hornsund (76°58.2N, 15°34.5E, *in situ* temperature 2.6°C) and Storfjord (77°33.0 N, 19°05.0 E, *in situ* temperature -1.7°C) were sampled on a cruise in September/October 1995. For further information about sampling sites, see Kostka *et al.* (1999). Sediment was collected with a multicorer, and one individual core was subsampled for enumeration of sulfate reducers by triplicate MPN series (American Public Health Association, 1969), sulfate reduction rate measurements with the whole core method (Jørgensen, 1978), and nucleic acid

analysis (Sahm *et al.*, 1999a; referred to as core A). The subcores were sliced on the ship and samples from five sediment layers between the surface and 30 cm depth (Fig. 1) were transferred to liquid medium (Knoblauch *et al.*, 1999b) containing either lactate (20 mM) or acetate (15 mM). Additionally, single dilution series with propionate (20 mM), or propanol (20 mM) were inoculated. The cultures were incubated at 4,10 and 20°C in our laboratory and growth of SRB was measured via sulfide production during the following 30 months.

At both sampling sites, maximum MPN counts of SRB occurred in the top 6 cm of the sediment. In particular in Storfjord, the highest sulfate reduction rates occurred deeper than the maximum cell counts (Fig. 1). Below that depth, cell numbers decreased sharply. Maximum cell numbers were generally detected on lactate as substrate in MPN series incubated at 10°C (Fig. 1b+d). Higher cell numbers at 10 than at 20°C indicate that the majority of cultivable sulfate reducers in the sediment are unable to grow at 20°C, thus providing the first microbiological evidence for a predominantly psychrophilic SRB community in a marine sediment. Maximum cell numbers on acetate were 10-100 fold lower than on lactate and were always highest at 20°C. These results are probably due to extremely slow growth of acetate oxidizers at 4 and 10°C, and not to a mesophilic acetate oxidizing SRB community. This conclusion is supported by the fact that the first positive enrichments of Storfjord, incubated at 4 and 10°C on acetate, were detected after more than six months and that counts increased slowly during the following two years.

In contrast to this microbiological evidence for a community with a psychrophilic growth potential (T_{opt} below 20°C), Sagemann *et al.* (1998) measured highest SRR in Hornsund and Storfjord sediments at 27°C. These process rate measurements seem to contradict our results from MPN counts. However, Isaksen and Jørgensen (1996) demonstrated that a moderately

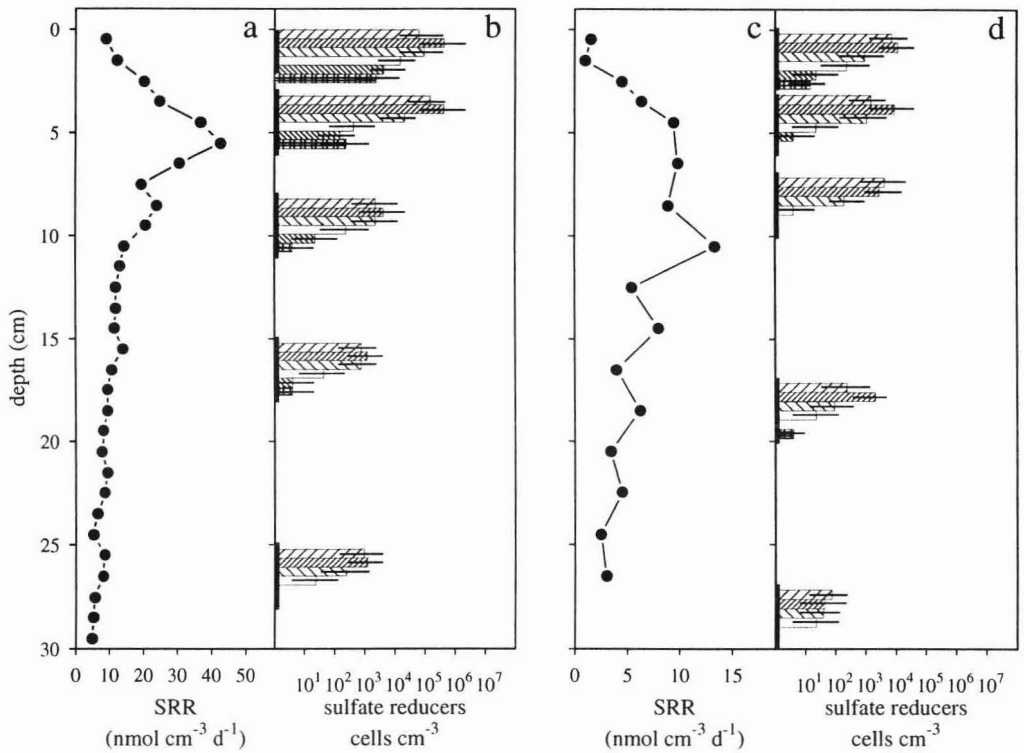

Fig. 1. Depth profile of sulfate reduction rates in Hornsund (a) and Storfjord (c) at in situ temperatures and MPN counts of sulfate reducers in Hornsund (b), and Storfjord (d) sediment. MPN series were incubated at different temperatures and with either lactate (▨ 20 °C, ▨ 10 °C, ▨ 4 °C) or acetate (□ 20 °C, ▨ 10 °C, ▦ 4 °C). Horizontal bars represent 95 % confidence intervals, vertical bars the sediment depth used for MPN enrichments.

psychrophilic SRB had an optimum temperature for sulfate reduction (28°C) 10°C higher than for growth (18°C). This result indicates that maximum sulfate reduction rates at 27°C in the Svalbard sediments might still be assigned to a psychrophilic community.

MPN counts yielded no evidence for a higher community size of cultivable sulfate reducers in Arctic sediments relative to temperate sediments since maximum cell counts of e.g. $4.3 \cdot 10^5$ cells cm^{-3} in Hornsund (Fig. 1b) are in the range of those reported previously for temperate marine sediments ($2 \cdot 10^5$-$2 \cdot 10^6$ cells cm^{-3} [Jørgensen and Bak, 1991; Lillebæk, 1995; Teske *et al.*, 1996]). Furthermore, parallel slot-blot hybridizations indicate that SRB numbers in Hornsund and Storfjord are comparable to those in temperate sediments (Sahm *et al.*, 1999a; Sahm *et al.*, 1999b). If the community size and the sulfate reduction rates in Arctic and temperate habitats are similar, then sulfate reduction rates per cell must be comparable too, irrespective of the temperature difference.

To test this possibility, pure cultures of Arctic SRB were isolated from the highest dilution steps of the MPN enrichments by the modified deep agar dilution technique (Isaksen and Teske, 1996). At 20°C, only three pure cultures could be isolated because most enrichments did not continue to grow after a transfer to fresh medium. None of these isolates is able to grow at the *in situ* temperature of the sampling sites, providing further evidence that the community active in the sediments is psychrophilic. At 4 and 10°C, thirty different strains were isolated from the MPN-enrichments. Based on a preliminary physiological and phylogenetic characterization, 19 psychrophilic strains were selected for further studies. All strains, except LSv22, had a T_{opt} below 20°C and only three isolates grow at 26°C (Table1). More relevant, however, is that they are the first isolates that grow at a typical temperature for polar sediments, the freezing point of seawater, -1.8°C (Table 1). Doubling times at -1.8°C were 4-6 days in case of lactate-grown strains LSv54, LSv514 and LSv21, but more than five weeks in case of acetate- and propionate-grown strains ASv26 and PSv29 (Knoblauch and Jørgensen, 1999).

To compare SRR of psychrophiles and mesophiles at the temperatures of their respective habitats, the specific SRR of psychrophilic SRB were measured

at the *in situ* temperature of the Arctic sediments (2.6 and -1.7°C) and rates of 9 mesophiles were measured at 4, 8 and 13°C, a temperature range normally encountered in temperate sediments. All cultures were grown to the exponential growth phase and rates were measured with the radiotracer method as described elsewhere (Knoblauch and Jørgensen, 1999). Specific SRR of psychrophiles at 2.6 and -1.7°C varied between 1 and 42 fmol cell^{-1} d^{-1} (Table 1). All mesophiles

Table 1. Growth characteristics and specific sulfate reduction rates of psychrophilic SRB measured at the *in situ* temperature of their habitat.

	Substrates[a]	incubation temperature (°C)	Specific SRR (±SD, n=3) (fmol cell^{-1} day^{-1})	growth at (°C) -1.8	4	15	20	26
Hornsund strains								
LSv20	lactate	2.6	14.0 ± 0.6	+	+	+	+	-
LSv21	lactate	2.6	2.7 ± 0.7	+	+	+	+	-
LSv22	lactate	2.6	13.0 ± 2.0	+	+	+	+	+
LSv23	lactate	2.6	2.3 ± 0.6	+	+	+	+	-
LSv24	lactate	2.6	11.0 ± 0.8	+	+	+	+	-
LSv25	lactate	2.6	2.8 ± 1.1	+	+	+	+	-
LSv26	lactate	2.6	6.9 ± 0.5	+	+	+	+	+
LSv27	lactate	2.6	2.6 ± 0.3	+	+	[b]n.d.	-	-
LSv28	lactate	2.6	2.6 ± 0.2	+	+	+	-	-
PlSv28	propanol	2.6	2.5 ± 1.4	+	+	+	-	-
PSv29	propionate	2.6	41.9 ± 23.4	+	+	-	-	-
ASv25	acetate	2.6	25.3 ± 0.3	+	+	+	+	-
ASv26	acetate	2.6	3.8 ± 1.0	+	+	-	-	-
ASv28	acetate	2.6	11.3 ± 0.9	+	+	+	+	+
		Hornsund mean:	10.2					
Storfjord strains								
LSv514	lactate	-1.7	3.6 ± 0.4	+	+	+	+	-
LSv52	lactate	-1.7	7.6 ± 3.7	+	+	+	+	-
LSv53	lactate	-1.7	0.9 ± 0.4	+	+	+	+	-
LSv54	lactate	-1.7	1.9 ± 0.2	+	+	+	-	-
LSv55	lactate	-1.7	6.2 ± 0.8	+	+	+	-	-
		Storfjord mean:	4.0					

[a] carbon substrates used for isolation and for measurements of specific sulfate reduction rates
[b] n.d. = not determined

Table 2. Specific sulfate reduction rates of mesophilic sulfate reducing bacteria at different temperatures

strain	DSMZ[a] number	Substrate[b]	specific sulfate reduction rate (± SD, n=3)[c] (fmol cell^{-1} day^{-1}) 4 °C	8 °C	13 °C
Desulfobacter postgatei	2043	acetate	11 ±1.6	19 ±1.4	38 ±5.9
Desulfobacter hydrogenophilus	3380	hydrogen	8.0 ±0.3	7.8 ±2.8	20 ±3.3
Desulfobulbus sp. 3pr10	2058	propionate	4.2 ±0.1	6.2 ±0.36	12 ±0.6
Desulfovibrio salexigens	2636	lactate	0.7 ±0.06	1.4 ±0.07	3.9±0.4
Desulfovibrio vulgaris	1744	lactate	0.4 ±0.05	0.8 ±0.06	2.1±0.1
Desulfobacterium autotrophicum	3382	lactate	1.6 ±0.07	2.9 ±0.2	4.4±0.4
Desulfofustis glycolicus	9705	glycolate	0.3 ±0.01	0.5 ±0.06	1.1±0.1
Desulfococcus niacini	2650	nicotinate	1.2 ±0.05	2.0 ±0.24	4.0±0.7
Desulfosarcina variabilis	2060	benzoate	0.7 ±0.4	9.0 ±2.3	20 ±0.6
		mean values	3.1	5.6	11.7

[a]All strains were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany
[b]Carbon substrates used for isolation and for measurements of specific sulfate reduction rates
[c]measurements of specific SRR were made in 15 ml Hungate tubes, except for *Desulfobacter hydrogenophilus*, that was incubated in flat 50 ml culture flasks to enhance hydrogen diffusion into the aqueous phase

reduced sulfate at 4 °C, although only *Desulfobacter hydrogenophilus* was able to grow at that temperature. Specific SRR of all mesophiles, except *Desulfobacter hydrogenophilus* (Table 2), increased exponentially with increasing temperatures, but were still comparable to those found in the psychrophiles at 6-10°C lower temperatures. Since it is difficult to directly compare rates of mesophiles and psychrophiles at low temperatures because their growth temperature range do not overlap, we fitted mean rates of mesophiles by the Arrhenius equation: Rate = $A \cdot \exp(-E_a \cdot [R \cdot T]^{-1})$, where A = a constant, E_a = apparent activation energy, R = gas constant and T = absolute temperature in K. The fit was extrapolated to <0°C, and compared to rates of psychrophiles (Fig. 2). Calculated rates for mesophiles at 2.6 and -1.7°C were

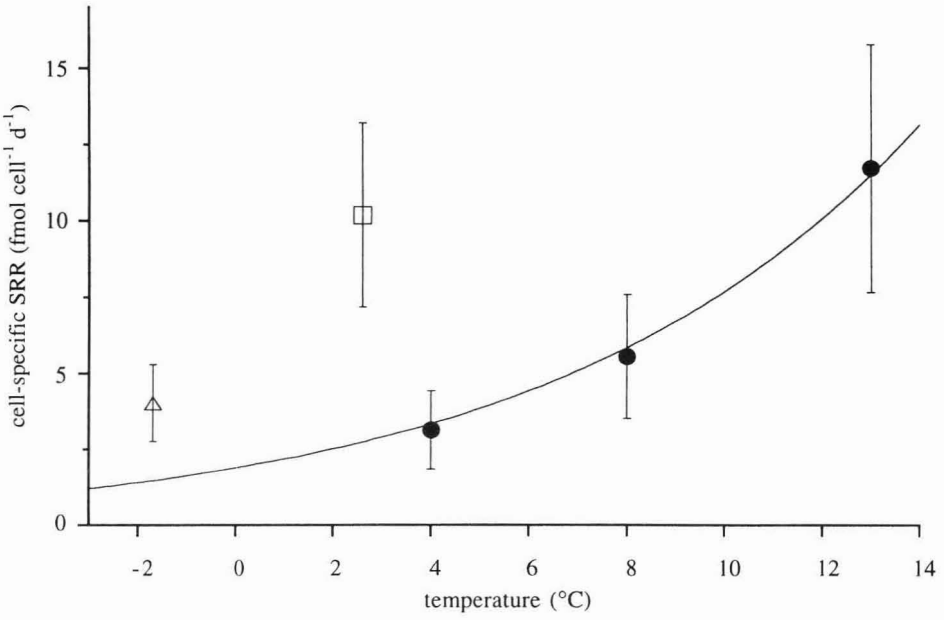

Fig. 2. Mean values of specific sulfate reduction rates of 10 mesophilic sulfate reducers (closed circles) determined at 4, 8 and 13 °C, 14 psychrophiles from Hornsund sediments (open square), and 5 psychrophiles from Storfjord sediments (open triangle). Dashed line represents the Arrhenius fit of specific sulfate reduction rates for mesophiles. Bars represent standard deviation of the mean of all strains.

3-4 fold lower than the measured rates for psychrophiles at the same temperatures (Fig. 2). The comparison of biomass specific SRR yielded similar differences (data not shown). These differences indicate that psychrophilic SRB are adapted to low temperatures not only because their minimum growth temperatures are at or below *in situ* temperatures, but also because their metabolic rates are comparable to those of mesophiles at 6-10°C higher temperatures. Many studies have demonstrated that organisms active at low temperature differ physiologically from their counterparts in warmer environments (Russel and Hamamoto, 1998, and references therein). Cellular membranes of psychrophiles tend to contain more unsaturated fatty acids (Chan

et al., 1971; Bhakoo and Herbert, 1979) and short chain fatty acids (Bhakoo and Herbert, 1979) than membranes of mesophiles. Changes in the membrane composition might lead to a more efficient solute uptake at low temperatures (Russell, 1990). Furthermore, psychrophiles synthesize enzymes with high catalytic activity at low temperatures (Feller *et al.*, 1994b) and produce more enzymes when the temperature decreases (Feller *et al.*, 1994a). Different enzymes or enzyme levels could be one explanation for comparable sulfate reduction rates of psychrophiles and mesophiles at different temperatures.

The calculated activation energy (E_a) of mesophilic SRB was 90.6 kJ/mol, which is within the range of 23-132 kJ/mol determined previously for sulfate reduction in temperate sediments (Aller and Yingst, 1980; Crill and Martens, 1987; Westrich and Berner, 1988) and close to the values of 74 and 85 kJ/mol calculated from specific SRR between 0 and 30°C of a *Desulfovibrio desulfuricans* strain (Kaplan and Rittenberg, 1964). Thus, we suppose that the specific SRR measured in pure cultures are representative for mesophilic sulfate reducers of temperate sediments. However, it cannot be ruled out that measured rates of mesophiles were biased by the inability of most strains to grow at the low experimental temperatures. This problem could not be avoided in our use of culture collection strains because mesophilic marine sulfate reducers that are able to grow down to 0°C are almost unknown.

2.2.

Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine Arctic sediments

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Thirteen psychrophilic sulfate-reducing isolates from two permanently cold fjords of the Arctic island Spitsbergen (Hornsund and Storfjord), were phylogenetically analyzed. They all belong to the δ subclass of *Proteobacteria* and are widely distributed within this group, indicating that psychrophily is a polyphyletic property. A new 16S rRNA-directed oligonucleotide probe was designed against the largest coherent cluster of these isolates. The new probe, as well as a set of available probes, were applied in rRNA slot-blot hybridization to investigate the composition of the sulfate-reducing bacterial community in the sediments. rRNA related to the new cluster of incomplete oxidizing, psychrophilic isolates made up 1.4 and 20.9 % of eubacterial rRNA at Storfjord and 0.6 - 3.5 % of eubacterial rRNA at Hornsund. This group was the second most abundant group of sulfate reducers at these sites. DGGE/hybridization analysis showed bands identical to our isolates. The data indicate that the psychrophilic isolates are quantitatively important in Svalbard sediments.

INTRODUCTION

Low environmental temperatures characterize the habitat of many prokaryotes living in marine sediments since 90% of the sea floor has a temperature of less than 4 °C (Levitus and Boyer, 1994). While prokaryotic activity is commonly found to be lower during cold seasons in temperate environments (for review see Rivkin *et al.*, 1996), the current data suggests that in permanently cold habitats bacterial activity is comparable to temperate environments at the respective ambient temperature (Arnosti *et al.*, 1998; Rivkin *et al.*, 1996; Glud *et al.*, 1998; Sagemann *et al.*, 1998). Arnosti *et al.* (1998) determined the temperature dependence of microbial degradation of organic matter and showed that carbon turnover in the cold Arctic is not intrinsically slower than in temperate environments. Also Sagemann *et al.* (1998) and Glud *et al.* (1998) found rates of sulfate reduction and benthic carbon mineralization in Arctic sediments to be comparable to those of temperate or even tropical sediments. Optimal temperatures for polysaccharide hydrolysis, oxygen consumption (Arnosti *et al.*, 1998), and sulfate reduction (Sagemann *et al.*, 1998) in permanently cold sediments were significantly higher than the ambient temperature; however, the relative activity at low *in situ* temperature as compared to optimum activity was generally higher than in samples from temperate habitats. These observations indicate that the bacterial community in these Arctic sediments is adapted to cold temperature. However, little is known about the diversity and composition of prokaryotic communities in cold marine sediments; and only few cold-adapted psychrophilic isolates from these environments have been studied so far (for review see Russel and Hamamoto, 1998). In addition, few cultivation-independent studies have been conducted in these habitats (Sahm and Berninger, 1998).

The aim of our project, conducted in the context of the above-mentioned studies, was to characterize the sulfate-reducing bacterial community of

permanently cold habitats and to quantify the abundance of psychrophilic sulfate reducers. We chose two sites, off the coast of Spitsbergen (Hornsund and Storfjord), which are never exposed to temperatures higher than 3 °C. We concentrated on sulfate-reducing prokaryotes because sulfate reduction is a major process of carbon mineralization in marine sediments (Jørgensen, 1982). A set of probes is available for the main phylogenetic groups of Gram-negative mesophilic sulfate reducers (Devereux *et al.*, 1992), and the different phylogenetic groups can be defined by distinct physiological features (Widdel and Bak, 1992; Widdel and Hansen, 1992). In a related study MPN-counts and isolation of psychrophilic sulfate reducers were carried out to enumerate and identify the sulfate-reducing bacteria (SRB) (Knoblauch *et al.*, 1999a). A new oligonucleotide probe was designed to target the largest cluster of these isolates. This newly-developed probe was applied along with an established set of probes, to quantify sulfate reducer rRNA in the sediment. The presence of the isolates was further evaluated by DGGE-analysis. The results of this study will be discussed in relation to data from a 16S rDNA clone library presented in an accompanying paper (Ravenschlag *et al.*, 1999).

MATERIALS AND METHODS

Study site and sampling procedure. Our study was conducted as part of a research cruise in the Arctic Sea from Tromsø (Northern Norway) to Spitsbergen (Arctic Ocean) in September/October 1995. Sediments from 2 different stations (Hornsund [76°58.2N,15°34.5E] and Storfjord [77°33.0N, 19°05.5E]) were investigated. *In situ* temperatures and depths were 2.6°C and 155 m for Hornsund and -1.7°C and 175 m for Storfjord. Sediment samples were collected with a multicorer. Samples for MPN-dilutions (Knoblauch *et al.*, 1999a) and molecular analysis were taken from the same core. The individual subcores (our replicates A and B) derived from two different multicorer cores.

The sediments were anoxic below a depth of approx. 8 mm (Glud *et al.*, 1998). Five distinct vertical horizons of 2-3 cm thickness were sectioned from the upper 30 cm of each core. The sediment of each section was carefully homogenized, and subsamples of 1 or 2 cm^3 were immediately frozen in liquid N_2.

DNA extraction and amplification of 16S rDNA. After three cycles of freezing and thawing, DNA was extracted directly using the method of Zhou *et al.* (1996), which is based on lysis with a high salt extraction buffer and extended heating in the presence of sodium dodecyl sulfate (SDS) and hexadecyltrimethylammoniumbromide. Lysis efficiency was checked by DAPI staining. In general, at least 90 to 96% of the cells were lysed. The DNA could be used for PCR without further purification. Primers GM5clamp (*Escherichia coli* position 341-357) and 907R (Muyzer *et al.*, 1995) were used to amplify the variable regions V3-V5 of the 16S rDNA in a touchdown PCR, as described by Buchholz-Cleven *et al.* (1997). To amplify the nearly complete 16S rDNA, primers 8F/1492R (Buchholz-Cleven *et al.*, 1997) were used in a 35 cycle PCR with an annealing temperature of 40°C. Bovine serum albumin (final concentration 3 mg ml^{-1}) was added routinely to the PCR reactions to prevent interference by humic acids (Romanowski *et al.*, 1993).

16S rDNA sequencing. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used to directly sequence the purified PCR products. Sequencing reactions were analyzed on the Applied Biosystems 373S DNA sequencer. Both strands of the amplification products were sequenced using primers 8F, 787F, 787R, 1175R, 1099 F, 1492R (Buchholz-Cleven *et al.*, 1997). Primer nomenclature refers to 5'-ends of the respective target sites on the 16S rDNA according to the *E. coli* numbering of 16S rRNA nucleotides (Brosius *et al.*, 1981).

Phylogenetic analysis. The ARB program package and the ARB database (Strunk *et al.*, 1999) were used for phylogenetic analysis. Sequences were aligned to the 16S rRNA primary structures present in the ARB database by using the automatic aligner tool, and the results were corrected manually where necessary. Pairwise distance matrix analysis was performed with the 16S rRNA sequences taking only those positions into account that were present in both sequences. Phylogenetic trees were reconstructed for all available sequences from the δ subclass of *Proteobacteria*, and a selection of representatives for major groups outside this subclass was used as an outgroup. Only sequences with at least 1350 nucleotides were used. Tree topology was evaluated by using neighbor joining, maximum parsimony, and maximum likelihood algorithms on the full set of data or on a subset. Furthermore, filters were applied that excluded positions with less than 50% conservation within the δ subclass. Branching orders that were not supported by all methods are shown as multifurcation (Fig. 1).

Accession numbers. The 16S rDNA sequences have been deposited in the GenBank database. Accession numbers are AF099054-AF099065, AF136008.

Oligonucleotide probes. Oligonucleotides were purchased from Biometra, (Göttingen, Germany). The probe-target sequence of Sval428 is: 5' GTAAAATCCTGTCAGATGG 3' (*E. coli* numbering 428-446). Probes used and their specificity are shown in Fig. 1.

RNA extraction and slot-blot hybridization. Nucleic acids were isolated directly by bead-beating, phenol extraction and isopropanol precipitation as described by Sahm and Berninger (1998). Between 10 and 100 ng of RNA was blotted on nylon membranes (Magna Charge, Micron Separations, Westborough, USA) in triplicate and probed with radioactively-labeled oligonucleotides as described previously (Stahl *et al.*, 1988). Membranes were prehybridized at 40°C and washed at different temperatures depending on the dissociation temperature (Td) of the probes: 54°C (EUB338 [Amann *et al.*,

1990]), 45°C (687 [Devereux *et al.*, 1992]), 59°C (660 [Devereux *et al.*, 1992]), 46°C (804 [Devereux *et al.*, 1992]), or 52°C (Sval428). The Td for probe Sval428 was determined according to Raskin *et al.* (1994) with rRNA from strain LSv20 used as one mismatch control. Intensity of hybridization signal was measured with a Phosphor Imager (Molecular Dynamics, Sunnyvale, USA) and quantified as described by Sahm *et al.* (1999b) using the program ImageQuant (Molecular Dynamics). rRNA isolated from *Desulfovibrio salexigens* (DSM 2638), *Desulfobulbus elongatus* (DSM 2908), *Desulfococcus multivorans* (DSM 2059), and strain LSv23 served as standard for hybridization with the sulfate reducer specific probes.

DGGE and Southern hybridization analysis. DGGE was performed on a D-Gene system (Bio Rad, München, Germany) as described previously (Muyzer *et al.*, 1996; Muyzer *et al.*, 1998). PCR products were analyzed directly on a 1-mm-thick 6% polyacrylamide gel containing a denaturing gradient from 20-80%. Electrophoresis using 1xTAE buffer (40 mM Tris-acetate,1mM EDTA; pH 8) was performed at 100 V for 20 h. After electrophoresis, the gels were stained in ethidium bromide and photographed on an UV transilluminator. DGGE gels were blotted onto nylon membranes via electroblotting as described by Muyzer *et al.* (1996). Hybridization analysis was performed with probe Sval428 using the protocol described by Santegoeds *et al.* (1998). The probe was end labeled with [γ-^{32}P]ATP, and the membrane was hybridized at 40°C over night. Stringent washes were performed in 2xSSC, 0.1% SDS (w/v) at the previously determined dissociation temperature of 56°C. Dissociation temperature of the probe was determined with the method described by Raskin *et al.* (1994) for DNA and RNA as targets. The hybridized membranes were sealed in plastic bags and exposed for 1-7 days on an X-ray film or a Phosphor Imager screen.

RESULTS AND DISCUSSION

Phylogenetic affiliation of isolates. Psychrophilic sulfate reducers isolated from MPN-enrichments (Knoblauch *et al.*, 1999a) were phylogenetically analyzed by 16S rDNA sequencing. All isolates belong to the δ subclass of the *Proteobacteria,* as it is the case for the majority of mesophilic sulfate reducers (Stackebrandt *et al.*, 1995). Adaptation to cold temperatures as represented by our sulfate-reducing isolates was widely spread within this phylogenetic group (Fig. 1). On the basis of their phylogenetic distance, strains ASv25, BSv41, and LSv20 belong to the existing genera *Desulfobacter sp.*, *Desulfobacterium sp.*, and *Desulforhopalus sp.* (3.6, 1.9, and 0.7% phylogenetic distance respectively) (Fig. 1). The remaining ten strains were only distantly related (6.4-11.6% phylogenetic distance) to known sulfate reducers. Five of these have recently been described as members of three new genera (Knoblauch *et al.*, 1999b). The results suggest that psychrophily is a polyphyletic property within the Gram-negative sulfate reducers. Furthermore, the wide distribution of our isolates within the δ Proteobacteria indicates that the diversity among psychrophilic SRB might be as high as among mesophiles.

Probe design. Since cultivation is inherently selective and often leads to the isolation of quantitatively less important microbial groups, we estimated abundance of these isolates with a cultivation independent method, i.e., rRNA slot-blot hybridization. Besides LSv55, all strains that were related to the *Desulfobacteriaceae* were targeted by an already existing probe (804) designed by Devereux *et al.* (1992). Of the remaining strains, six incomplete oxidizers formed a cluster related to *Desulfocapsa sp.*, *Desulfofustis glycolicus*, and the only other known moderately psychrophilic sulfate reducer "*Desulforhopalus vacuolatus*" ltk10 (Isaksen and Teske, 1996) (Fig. 1). Two strains of this cluster have recently been described as species of the new genus, *Desulfotalea*

sp. (Knoblauch *et al.*, 1999b). The cluster will in the following be referred to as *Desulfotalea*-cluster. This cluster was chosen to design a new oligonucleotide probe (Sval428). In addition to the two *Desulfotalea* strains and strains LSv23, LSv24, and LSv53, the probe targets one of the mesophilic sulfate reducers

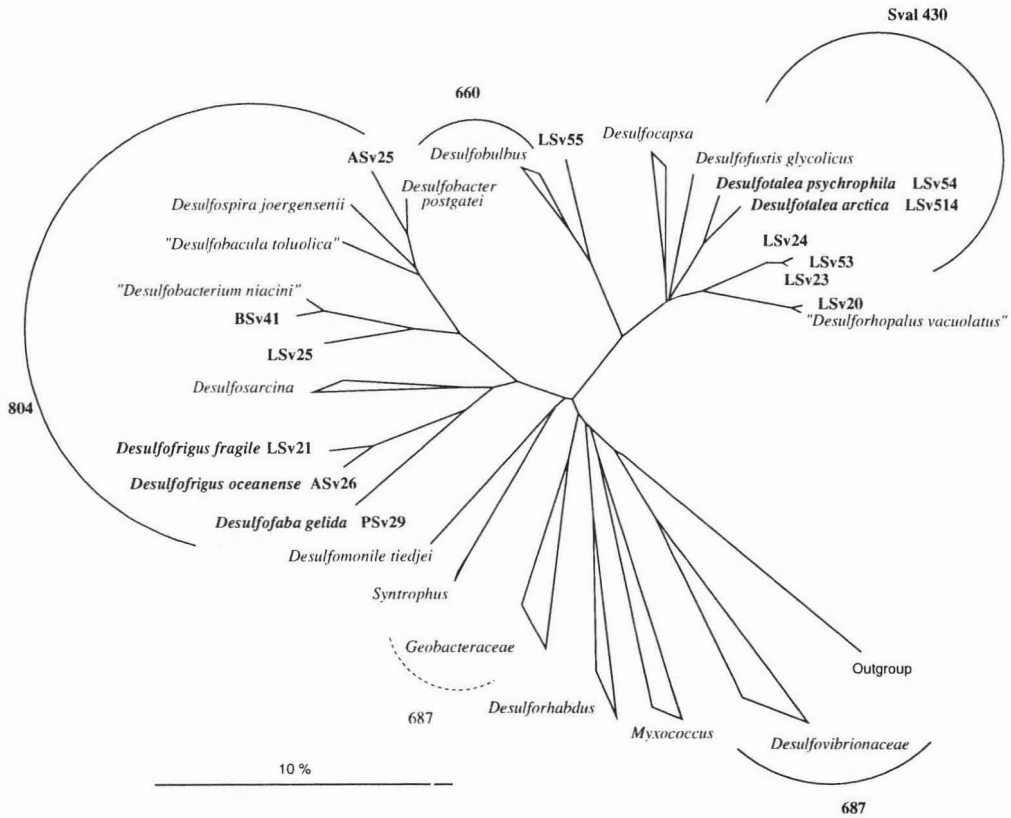

Fig. 1. Phylogenetic affiliation of psychrophilic sulfate-reducing isolates and specificity of probes used in this study. The tree shows the δ subclass of Proteobacteria and was constructed using Neighbour Joining and a 50 % conservation filter. The arcs comprise the respective probe target groups. Sval428: described in this study. Other probes described by Devereux et al. (1992). Strains isolated from Svalbard sediments are shown in bold face. Strain names indicate the carbon source on which they were isolated. A: acetate, L: lactate, P: propionate, B: betaine, and the sampling site (Sv2: Hornsund, Sv5: Storfjord).

Desulfofustis glycolicus (Friedrich *et al.*, 1996) (Fig. 1). It has one mismatch to LSv20 and "*Desulforhopalus vacuolatus*" ltk10 (Isaksen and Teske, 1996). LSv55 and LSv20 are the only strains isolated from Spitsbergen sediments not targeted by the set of probes used.

rRNA quantification of sulfate reducers and estimation of community size. rRNA slot-blot hybridization revealed that concentration of SRB-rRNA for all target-groups generally decreased with depth (Table 1), following the trend of total prokaryotic rRNA (Sahm and Berninger, 1998) and MPN-counts (Knoblauch *et al.*, 1999a). The relative contribution of SRB rRNA to total eubacterial rRNA (pooled signals from all probes), however, increased from 3.8/4.0% (coreA/B) in the surface to 10.5/17% at a depth of 15-18 cm in Hornsund and from 10.5/7% to 58.6/36.4% at a depth of 27-30 cm in Storfjord (Table 2).

The high concentration of detectable SRB rRNA in the first 2-3 cm of the sediment is in striking contrast to the very low sulfate-reduction rates measured

Table 1. Recovered SRB rRNA

Depth interval (cm)	Total SRB rRNA (ng cm^{-3} sediment)	
	core A	core B
Hornsund		
0-2	113	134
3-6	71	81
8-11	26	55
15-18	8	43
25-28	4	8
Storfjord		
0-3	87	36
3-6	82	25
7-10	21	17
17-20	15	13
27-30	10	12

within this layer in parallel cores (Sagemann *et al.*, 1998; Knoblauch *et al.*, 1999a). High abundance of SRB in the zone where little sulfate reduction rates are measured can also be seen in MPN-counts (Knoblauch *et al.*, 1999a) and was also observed in a coastal sediment of the Baltic Sea by RNA slot-blot hybridization (Sahm *et al.*, 1999b). These findings suggest that SRB might use electron acceptors other than sulfate in the oxidized zone of the sediment. In this respect it is interesting to note that four of the five isolated strains tested for iron reduction were able to grow on Fe(III) (Knoblauch *et al.*, 1999a). In addition Kostka *et al.* (1999) could show that in Storfjord sediments Fe(III) reduction accounted for almost 10% of total carbon oxidation, while it was insignificant at Hornsund. Other potential electron acceptors like NO_3^- or Mn(IV) were of minor importance in the investigated sediments.

Table 2. Relative contribution of different probe target groups to the total eubacterial rRNA

	% of bacterial rRNA (core A / core B)				
Depth interval (cm)	*Desulfotalea* Sval428	*Desulfovibrio-naceae* 687	*Desulfobulbus sp.* 660	sum of detected SRB	% Sval430 of SRB (mean)
Hornsund					
0-2	0.6 / 1.1	2.5 / 2.3	0.7 / 0.6	3.8 / 4.0	22
3-6	0.7 / 2.5	4.6 / 3.6	1.2 / 1.5	6.5 / 7.6	22
8-11	0.6 / 3.5	6.4 / 10.1	1.6 / 1.4	8.6 / 15.0	15
15-18	0.3 / 2.6	8.6 / 12.9	1.6 / 1.5	10.5 / 17.0	9
25-28	0.0 / 1.9	6.0 / 11.1	1.2 / 0.5	7.2 / 13.5	13
Storfjord					
0-3	1.6 / 1.4	8.1 / 5.0	0.8 / 0.6	10.5 / 7	18
3-6	2.6 / 3.5	14.9 / 6.5	2.7 / 0.8	20.2 / 10.8	23
7-10	3.9 / 4.4	10.4 / 6.7	1.4 / 0.7	15.7 / 11.8	31
17-20	11.5 / 9.2	20.7 / 14.1	1.5 / 0.9	33.7 / 24.2	36
27-30	20.9 / 15.0	36.0 / 20.4	1.7 / 1.0	58.6 / 36.4	38

Relative abundance of SRB and physiological considerations. SRB can, in general, be divided in two major groups: those that oxidize the carbon source completely to CO_2 and those that oxidize the carbon source incompletely to acetate. rRNA hybridization revealed a predominance of incomplete-oxidizing SRB in these sediments. We detected incomplete oxidizing groups targeted by probes 687 (*Desulfovibrionaceae* but also some *Geobacteraceae* [Lonergan *et al.*, 1996]), 660 (*Desulfobulbus sp.*), and Sval428 (*Desulfotalea*-cluster), while the complete oxidizing genera *Desulfococcus sp.*, *Desulfosarcina sp.*, *Desulfobacterium sp.*, *Desulfobacter sp.* targeted by 804 were below the detection limit (Table 2). This result is in agreement with cultivation-dependent data since cell numbers of complete oxidizers on acetate were 10- to 100-fold lower than lactate oxidizers (Knoblauch *et al.*, 1999a). If this finding reflects the actual relation between complete and incomplete oxidizers, the RNA concentration for acetate oxidizers would be at or below the detection limit of slot-blot hybridization. Another possible explanation for not detecting RNA of complete oxidizers could be that these organisms were not targeted by the probe used; all complete oxidizing strains isolated in this study, however, had the target sequence for probe 804.

The low abundance of complete oxidizers contrasts with results from estuarine, coastal, and vegetated salt marsh sediments (Devereux *et al.*, 1996; Sahm *et al.*, 1999b; Rooney-Varga *et al.*, 1997) where the 804 target group was reported to be one of the major groups of SRB. Dominant sulfate reducers in these studies were the nutritionally versatile genera *Desulfobacterium sp.*, *Desulfococcus sp.*, and *Desulfosarcina sp.* The occurrence of these groups may possibly reflect an input of a wide variety of carbon sources in coastal zone habitats close to the mainland. Our study, in contrast, was conducted in a remote, sparsely populated region. Data on the amount and type of biologically-available carbon sources would be necessary to determine the relationship between substrates and occurrence of specific groups of SRB.

A second group of complete oxidizers is of special interest in sediments: namely, those that grow readily on acetate. These bacteria belong to the genus *Desulfobacter sp.*, like strain ASv25, or to the Gram-positive *Desulfotomaculum sp.* Although acetate is hypothesized to be one of the major carbon sources for SRB in marine sediments (Sørensen *et al.*, 1981; Parkes *et al.*, 1989), we could not detect *Desulfobacter sp.*-rRNA in our samples. Boschker *et al.* (1998) showed recently that addition of ^{13}C-acetate to an intertidal sediment led to an incorporation of label in polar lipid-derived fatty acids typical of *Desulfotomaculum acetoxidans*. A rRNA-probe for *Desulfotomaculum sp.* is not yet available. Neither cultivation (Knoblauch *et al.*, 1999a) nor clone library data (Ravenschlag *et al.*, 1999) indicate the presence of *Desulfotomaculum sp.*, but the use of a specific probe is needed to further investigate their role in acetate oxidation in marine sediments.

The major group of SRB was the *Desulfovibrionaceae/Geobacteraceae* cluster (probe 687) which, in the deeper zones, accounted for up to 8.6/12.9% of the RNA at Hornsund (15-18 cm) and for 36.0/20.4% at Storfjord (27-30 cm) (Table2); however, no *Desulfovibrionaceae* were isolated from MPN-cultures (Knoblauch *et al.*, 1999a). We took this as an indication that the detected RNA might be coming from organisms of the *Geobacteraceae*-group. A clone library established for Hornsund sediment samples (see accompanying publication [Ravenschlag *et al.*, 1999]) further supported this theory. Of all clones screened, 46 (13%) gave a positive signal with probe 687. Diversity within this group was very low, with one phylotype being represented by 39 clones and six additional phylotypes only represented by one or two clones (Ravenschlag *et al.*, 1999). All belonged to the family *Geobacteraceae* and were most closely related to *Desulfuromonas palmitatis*. Species of the genus *Desulfuromonas* belong to the δ subclass of *Proteobacteria* and are able to completely oxidize acetate via reduction of sulfur (Widdel and Pfennig, 1992). Since complete-oxidizing genera of sulfate reducers (804 target group) were

below the detection limit, the high abundance of *Desulfuromonas sp.* related sequences in the clone library might indicate that acetate is mineralized by sulfur reducers in these sediments; however, the phylogenetic distance between the clones and *Desulfuromonas palmitatis* is so large (6.3%) that we can only speculate on the possible physiological properties of this group until pure cultures have been isolated. Sequence information derived from the clone library will enable us now to design a specific probe for this 687-positive clone group and investigate its actual abundance. Furthermore the phylogenetic affiliation might help to choose selective culture conditions.

RNA related to the genus *Desulfobulbus sp.* (probe 660) was present in low amounts in both stations with a relative contribution varying between 0.5-2.7%. Clone library data suggest at least one additional group of SRB not targeted by our probes. This group is related to *Desulfobulbus sp.* and to isolate LSv55 and represents 6.5% of the clone library (Ravenschlag *et al.*, 1999). A new specific probe is currently being developed to investigate their abundance in the sediment.

Occurrence of the new psychrophilic isolates. The *Desulfotalea* cluster (probe 428), containing many psychrophilic isolates, was the second largest group among the detected sulfate reducers. In Storfjord, up to 15.0/20.9% (27-30 cm) of the eubacterial RNA was related to our isolates (Table 2). To estimate whether potentially dominant strains of the target group had been isolated, DGGE was performed on community DNA and analyzed by Southern hybridization with probe Sval428. Position of the hybridization signals within the community pattern was compared to the position of amplified 16S rDNA from isolates belonging to this group (Fig. 2). In both stations, we could detect several positive bands. One of them had the same position as isolates LSv23, LSv24, and LSv53 (Fig. 2). These isolates are closely related, showing 3 to 4

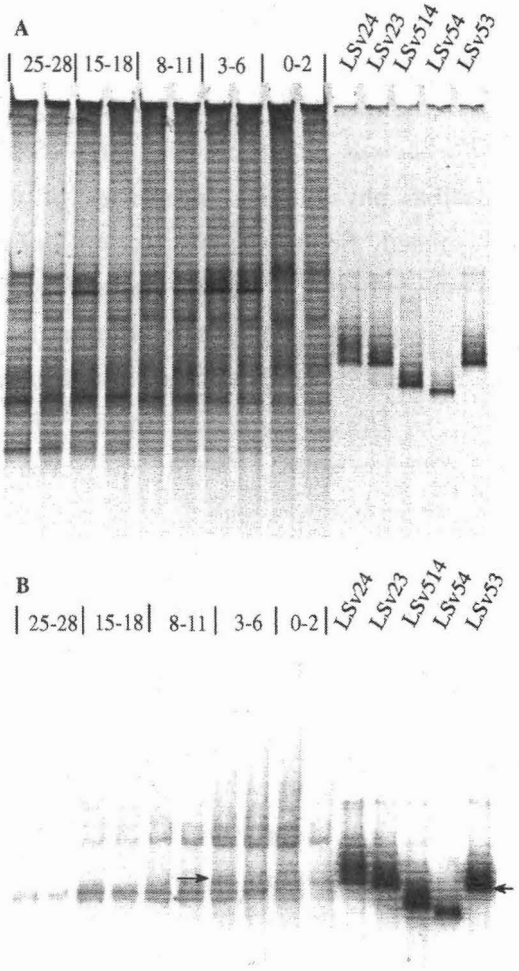

Fig. 2. DGGE-hybridization analysis of community 16S rDNA patterns with probe Sval428 for Hornsund sediments. (A) DGGE gel (B) corresponding hybridization results. Numbers give the depth range from which target DNA was extracted. For each depth two cores have been investigated. LSv***: DGGE-fragment of the psychrophilic sulfate-reducing isolates targeted by probe Sval428. Arrow indicates corresponding bands between community profile and isolates.

bases difference within the amplified DNA-fragment, and cannot be distinguished by DGGE. The presence of additional positive bands showed that there are at least three additional RNA-species of this group present in Hornsund (Fig. 2) and one in Storfjord sediments (data not shown). High abundance of *Desulfotalea*-related rRNA and the identification of bands corresponding to isolates in the community-DNA profile demonstrate that a quantitatively important group of sulfate reducers was isolated from Svalbard sediment. In addition, this group was the second most abundant of the detected SRB (Table 2). Since it is doubtful that the most abundant SRB-group, target group 687, is really a group of sulfate reducers, the *Desulfotalea*-cluster, containing mainly psychrophilic strains, may even be the most abundant of SRB. This observation relates to the question whether the bacterial community in Arctic sediments consists of cold adapted prokaryotes. Our results show that a major group of SRB in this habitat is psychrophilic.

In the present study, we showed that psychrophilic SRB are related to mesophilic strains and probably as phylogenetically diverse as the mesophiles. A new group of psychrophilic sulfate-reducing isolates is abundant in the sediments from which they were isolated. Their abundance suggests that they may play a previously-unrecognized role in the sulfur cycle of marine sediments.

2.3.

Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov.

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Five psychrophilic Gram-negative sulfate-reducing bacteria were isolated from marine sediments off the coast of Svalbard. All isolates grew at the *in situ* temperature of -1.7 °C. In batch cultures, strain PSv29^T had highest growth rate at 7 °C, strains ASv26^T and LSv54^T at 10 °C and strains LSv21^T and LSv514^T at 18 °C. The new isolates used the most common fermentation products in marine sediments, such as acetate, propionate, butyrate, lactate and hydrogen, but only strain ASv26^T was able to oxidize fatty acids completely to CO_2. The new strains had growth optima at neutral pH and marine salt concentration, except for LSv54^T which grew fastest with 1 % NaCl. Sulfite and thiosulfate were used as electron acceptors by strains ASv26^T, PSv29^T and LSv54^T and all strains except PSv29^T grew with Fe^{3+} (ferric citrate) as electron acceptor. Chemotaxonomy based on cellular fatty acid pattern and menaquinones showed good agreement with the phylogeny based on 16S rRNA sequences. All strains belonged to the δ subclass of *Proteobacteria* but had at least 9 % evolutionary distance to known sulfate reducers. Due to the phylogenetic and phenotypic differences between the new isolates and their closest relatives we propose the establishment of the new genera *Desulfotalea* gen. nov., *Desulfofaba* gen. nov., and *Desulfofrigus* gen. nov., with strain ASv26^T as type strain of the type species *Desulfofrigus oceanense* sp. nov., LSv21^T as type strain of *Desulfofrigus fragile* sp. nov., PSv29^T as type strain of the type species *Desulfofaba gelida* sp. nov., LSv54^T as type strain of the type species *Desulfotalea psychrophila*, sp. nov., and LSv514^T as type strain of *Desulfotalea arctica* sp. nov.

INTRODUCTION

Sulfate reducers are responsible for up to 50% of the organic carbon remineralization in marine sediments (Jørgensen, 1982; Canfield *et al.*, 1993; Nedwell *et al.*, 1993). Acetate, propionate, lactate, butyrate and hydrogen, which are the major end products of fermentation, constitute their most important carbon and energy substrates (Sørensen *et al.*, 1981; Christensen, 1984; Parkes *et al.*, 1989). According to their nutrition, sulfate-reducing bacteria can be separated into two distinct groups. Lactate, hydrogen and propionate are the typical substrates for incompletely oxidizing sulfate-reducing bacteria which are mainly represented by *Desulfovibrio* and *Desulfobulbus* species. The main end product of their catabolism is acetate which they do not oxidize further. The major substrates of completely oxidizing sulfate-reducing bacteria like *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, and *Desulfosarcina* strains, are fatty acids which are oxidized to CO_2 (Holt *et al.*, 1994). Phylogenetically, most sulfate reducers belong to the δ subclass of *Proteobacteria*.

The natural environment of most sulfate reducers is cold, since 90% of the sea floor has temperatures below 4°C (Levitus and Boyer, 1994). Like other benthic bacteria, sulfate reducers must therefore be able to grow at low temperatures, however nearly all the known isolates are mesophiles with a temperature optimum at or above 30°C and unable to grow below 4°C (Widdel and Bak, 1992). It was unclear, whether those sulfate reducers active at low *in situ* temperatures are closely related to the known mesophiles or whether they represent members of new genera and species. Pure cultures were needed to understand their metabolism and temperature adaptation as well as their phylogeny. The first moderately psychrophilic sulfate-reducing species, "*Desulforhopalus vacuolatus*" was isolated by Isaksen and Teske (1996) from a temperate estuary. In polar environments with permanent temperatures around 0°C, low-temperature adapted bacteria should be the dominant organisms. The aim of the present study was to isolate and describe the most abundant

low-temperature adapted sulfate reducers from polar sediments. Special attention was paid to organisms oxidizing acetate, propionate, lactate and butyrate.

METHODS

Sources of organisms. Arctic marine sediments at Svalbard were sampled in 1995 on a cruise with the RV "Jan Mayen". Strains LSv21^T, ASv26^T and PSv29^T originated from Hornsund sediment (76°58.2 N, 15°34.5 E) with a bottom water temperature of 2.6°C. Strains LSv54^T and LSv514^T were isolated from Storfjord sediment (77°33.0 N, 19°05.0 E) with a bottom water temperature of -1.7°C. Further information about sampling sites are given in Glud *et al.* (1998). "*Desulforhopalus vacuolatus*" strain ltk10^T (DSM 9700) was kindly provided by Kai Finster, Århus, Denmark; *Desulfovibrio giganteus* (DSM 4123) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig.

Enrichment, isolation and cultivation. Sediment samples were collected with a multicorer and subsampled directly on deck of the ship at an ambient temperature of 2-7°C. Subcores were sliced in an anaerobic glove bag and samples from five sediment depths between the surface and 30 cm were transferred to 90 ml sterile artificial seawater medium. These samples were suspended for two minutes with a vortex mixer and further diluted in 15 ml Hungate tubes containing 10 ml medium (Widdel and Bak, 1992). The medium contained (in g l^{-1}): NaCl, 26.4; $MgSO_4 \cdot 7H_2O$, 6.8; $MgCl_2 \cdot 6H_2O$, 5.7; $CaCl_2 \cdot 2H_2O$, 1.5; KBr, 0.09; KCl, 0.7. After autoclaving, the medium was cooled under a gas mixture of CO_2/N_2 (10/90, v/v) and the following components were added: 50 ml of a NH_4Cl (5 g l^{-1}) and KH_2PO_4 (4 g l^{-1}) solution, 1 ml nonchelated trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin solution (modified solution 6, with an additional 4 mg folic acid and 1.5 mg lipoic acid

per 100 ml), 1 ml thiamin solution, 1 ml vitamin B_{12} solution, 1 ml riboflavin solution (25 mg l^{-1} in 25 mM phosphate buffer, pH 3.2), 30 ml bicarbonate solution (1 M), 1 ml resazurine solution (1 g l^{-1}) and 1 ml sodium sulfide solution (1 M). If necessary, the pH was adjusted with HCl or NaOH to 7.1-7.3. The medium was dispensed under an atmosphere of CO_2/N_2 (10/90, v/v) into sterile serum bottles that were closed with black butyl rubber stoppers or into sterile 15 ml Hungate tubes. Before inoculating the medium, dithionite (final concentration 150 µM) and the desired carbon source were added from sterile stock solutions. The dilution series were inoculated on board the ship and transported back at 4°C. In our laboratory they were incubated at five different temperatures between 0 and 20°C. For the isolation of pure cultures the modified deep agar dilution technique (Isaksen and Teske, 1996) was applied, which protects temperature sensitive organisms from overheating. Agar (Agar Noble, DIFCO, Detroit, USA) was washed five times with distilled water (Widdel and Bak, 1992) before use. After three to four subsequent agar dilution series, 30 different pure cultures were isolated from the 0, 4, and 10°C enrichments. Stock cultures were kept at the temperature used for isolation and transferred every three to four weeks to fresh medium. For the characterization of pure cultures, the saltwater medium (Widdel and Bak, 1992) with a lower concentration of major salts was used. This medium contained (in g l^{-1}): NaCl, 20; Na_2SO_4, 4; $MgCl_2 \cdot 6H_2O$, 3; $CaCl_2 \cdot 2H_2O$, 0.15; KBr, 0.09; KCl, 0.5. After autoclaving, the medium was prepared as described above. To prevent a damage of temperature sensitive cells, great care was taken to protect enrichments and pure cultures from temperatures above those used for isolation.

Physiology and metabolism. The salt requirement for growth was monitored in media with 15 different NaCl concentrations between 0.2 and 5.8% (w/v) or 16 different $MgCl_2 \cdot 6H_2O$ concentrations between 0.0 and 7.0% (w/v). The concentration of all other salts, except the one tested, were kept constant. The vitamin demand of the different strains was tested for at least ten subsequent

transfers on medium without vitamins. The pH optimum was tested using media adjusted to 12 different pH values between 4.9 and 9.1. The pH was adjusted in triplicate tubes with HCl or NaOH and the tubes were inoculated. The initial pH was measured in one tube and the remaining tubes were incubated. Sulfide was measured periodically during the following six months. Growth with different electron donors was tested with sulfate as electron acceptor. Tubes without electron donor were inoculated and served as negative controls. Sulfide was measured periodically during the following year. Growth tests on different electron acceptors were made in sulfate-free medium which was supplied with the carbon source used for isolation of the tested strain and either thiosulfate (10 mM), elemental sulfur, nitrate (5 mM), nitrite (2 mM), Fe(III)-oxohydroxide, or Fe(III)-citrate (30 mM). Amorphous Fe(III)-oxyhydroxide was prepared by titration of an acidic $FeCl_3$ solution (0.5 M) with NaOH (2 M) to pH 7.0. Elemental sulfur was added with a spatula from a sterilized suspension, all other electron acceptors were added from sterilized stock solutions. Tubes without electron donor served as negative controls. Disproportionation of thiosulfate and elemental sulfur was tested in sulfate free medium. Either 20 mM thiosulfate or elemental sulfur plus Fe(III)-oxyhydroxide were added to the tubes. Additionally 2 mM acetate was added as a carbon source. All test tubes were inoculated with a sulfate-free preculture. The same precultures were used as inoculum for fermentation tests. Test tubes contained no electron acceptor but either lactate, pyruvate, fumarate, malate, or propionate at a final concentration of 10 mM. Growth was measured by direct counts under the light microscope. All tests were incubated at least in duplicates at the temperature used for isolation of the different strains, 4 or 10°C.

Fatty acids, lipoquinones and polar lipids. Cellular fatty acids were determined at the DSMZ, Braunschweig, Germany by R. M. Kroppenstedt. Fatty acid methyl esters were obtained by saponification and separated using a gas

chromatograph as described by Vainshtein *et al.* (1992). Respiratory lipoquinones and polar lipids were extracted from freeze-dried cell material and analyzed by thin layer chromatography (Tindall, 1990). The analyses were carried out by B. J. Tindall at the DSMZ.

Pigments and Gram-staining. The desulfoviridin test was carried out as described by Postgate (1984). *Desulfovibrio giganteus* (DSM 4123) served as positive control. To determine the Gram-reaction of the strains, heat fixed cells were stained with crystal-violet as described in Murray *et al.* (1994).

G + C content of genomic DNA. The G + C content of the genomic DNA was determined by HPLC (Mesbah *et al.*, 1989) at the DSMZ.

Chemical analysis. Sulfide was measured with the quick method described by Cord-Ruwisch (1985). If a higher precision was needed, the methylene blue method of Cline (1969) was applied. Volatile fatty acids and lactate were determined by ion exclusion chromatography with a HPLC system (Sykam, Gilching, Germany) and a refractometer (ERC-7515, ERC. INC. Alteglofsheim, Germany) as detector. The components were separated on a Sarasep WA1 column (300 x 7.8 mm) at 60°C with H_2SO_4 (15 mM) as eluent. The flow was adjusted to 0.6 ml min^{-1}. 50 µl of a 0.45 µm filtered sample (Acrodisc 4, Gelman Sciences, Michigan, USA) were injected on the column. Dissolved Fe^{2+} was determined according to Stookey (1970) with a Ferrozine solution (1 g l^{-1} in 50 mM HEPES buffer, pH 7.0). A 100 µl sample was diluted in 5 ml HCl (0.5 M) and after 15 minutes 50 µl were removed and added to 2.5 ml of Ferrozine solution and measured in a spectrophotometer (UV-1202, Shimadzu, Duisburg, Germany) at a wavelength of 562 nm. Organic carbon was determined in a CHNS analyzer (Cutter and Radford-Knoery, 1991). A known culture volume was filtered on two GF/F-filters (1.28 cm, Frisenette, Ebeltoft, Denmark) placed in two filter holders on top of each other. The lower filter was used as a blank. The filters were flushed with a marine salt solution and then dried in a stream of sterile-filtered air. The filters were placed in tin capsules

and 50 µl distilled water and 50 µl HCl (50 mM) were added to dissolve the bicarbonate. After two hours the filters were dried over-night at 105°C and analyzed on a CHNS analyzer (NA1500N, Fisons, Rodano, Italy)

Growth determination and growth rates. Since most of the isolates tended to grow in aggregates the determination of growth via an increase of the optical density (OD) was difficult. Growth was therefore measured routinely by the increase of sulfide in the cultures. Sulfide concentrations correlated significantly with bacterial cell numbers in the cultures ($P < 0.005$). If direct cell counts or OD were needed, cultures were carefully homogenized with an ultrasonic probe (HD200, Bandelin, Berlin, Germany) applying the lowest intensity. Microscopic controls revealed that cells were not damaged by this treatment. Direct cell counts were made under the microscope in an improved Neubauer chamber. The OD was measured in a spectrophotometer (UV-1202, Shimadzu, Duisburg, Germany) at 580 nm. Growth rates were calculated in exponentially growing cultures by a linear regression of $\ln OD_{580}$ as a function of time.

DNA isolation and DNA-DNA hybridization. DNA of strains LSv514^T, LSv54^T, and "*Desulforhopalus vacuolatus*" ltk10^T was isolated according to Marmur (1961). DNA-DNA hybridization was performed using the hydroxylapatite method as described by Ziemke *et al.* (1998) with the exception that DNA was labeled with ^{32}P-dCTP by nick translation as described by Rossello-Mora *et al.* (1994). After denaturation, DNA-DNA mixtures were incubated at 30°C below the melting temperature of homologous DNA, which in our case was 62°C for all hybridization pairs. The degree of reassociation (binding ratio) was calculated by dividing the counts for double stranded DNA by the total counts for double and single stranded DNA. The relative binding ratio for the heterologous pairs is expressed as the percentage of homologous binding (Lind and Ursing, 1986).

16S rDNA amplification. Cells were harvested from 2 ml culture samples by centrifugation and resuspended in 100 µl of 1 x PBS. A subsample of 1 µl was used directly as template for the amplification of 16S rDNA. PCR reactions were performed as described by Buchholz-Cleven *et al.* (1997). To amplify the nearly complete 16S rDNA, primers 8F/1492R (Buchholz-Cleven *et al.*, 1997) were used in a 35 cycle PCR with an annealing temperature of 40°C.

16S rDNA sequencing. PCR products were purified with the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany). The Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used to directly sequence the purified PCR products. Sequencing reactions were analyzed on the Applied Biosystems 373S DNA sequencer. Both strands of the amplification products were sequenced using primers 8F, 787F, 787R, 1175R, 1099F, 1492R (Buchholz-Cleven *et al.*, 1997). Primer nomenclature refers to 5'-ends of the respective target sites on the 16S rDNA according to the *E. coli* numbering of 16S rRNA nucleotides (Brosius *et al.*, 1981).

Phylogenetic analysis. The ARB program package and the ARB database (Strunk *et al.*, 1999) were used for phylogenetic analysis. Sequences were aligned to the 16S rRNA primary structures present in the ARB database by using the automatic aligner tool and the results were corrected manually where necessary. Pairwise distance matrix analysis was performed with the 16S rRNA sequences taking only those positions into account that were present in both sequences. Evolutionary distances were calculated using the Jukes-Cantor correction. Phylogenetic trees were reconstructed with representatives of most genera from the δ subclass of *Proteobacteria*. A selection of representatives for major groups outside this subclass was used as an outgroup. Only sequences with at least 1400 nucleotides were used. Tree topology was evaluated by using neighbour joining, maximum parsimony, and maximum likelihood algorithms on either the full set of data or on a selected subset. Furthermore, filters were applied that excluded positions with less than 50% conservation within the δ

subclass. Branching orders that were not supported by all methods are shown as multifurcations.

Naming of strains. The isolated strains were named with a code indicating the carbon source used for isolation (L = lactate, A = acetate, P = propionate), the sampling site (Sv2 = Hornsund, Sv5 = Storfjord) and a number.

RESULTS

Enrichment and isolation. Enrichment cultures at 4 and 10°C started to produce sulfide after 4 weeks if lactate was used as carbon source, and after 8 to 10 weeks if acetate or propionate was used. Sulfide producing enrichments were transferred to fresh medium until stable enrichments were obtained and subsequently agar dilution series were inoculated. After six to eight weeks all dilution series were dominated by brownish to blackish disk-shaped, smooth colonies. If lactate was used as carbon source, also white, fluffy colonies were present in the lower dilutions. Single colonies were picked and directly transferred to new dilution series until pure cultures were obtained. Only the brownish colonies contained sulfate reducers. Thirty pure cultures were isolated from different sediment samples and dilution steps. Based on a preliminary physiological and phylogenetic characterization, the five psychrophilic strains presented here were selected for further description. Strains LSv21^T, LSv54^T and LSv514^T were isolated on lactate, whereas acetate was used for the isolation of ASv26^T, and propionate for the isolation of PSv29^T. The temperature during isolation was 4°C for ASv26^T, LSv21^T, PSv29^T and LSv514^T and 10°C for strain LSv54^T.

Purity controls. All strains were transferred on media containing yeast extract (0.1% w/v) and either formate (20 mM), pyruvate (10 mM), glucose (5 mM), fructose (5 mM), or the carbon source used for isolation of the respective strain.

Microscopic examinations revealed that no morphologies different from the tested strains could be detected in any culture. Growth on yeast extract, glucose or fructose was never observed. Additionally the different strains were transferred to agar dilution series. Only the strain typical colony morphologies developed in all dilution steps.

Morphology. Cells of the acetate oxidizing strain ASv26^T (Fig. 1a) were thick rods with rounded ends. Growth in loose clumps was often observed. Cells of strain LSv21^T (Fig. 1b) were slightly curved rods with rounded ends. PSv29^T (Fig. 1c) was a large, slightly curved, peanut shaped rod. The cells grew exclusively in dense aggregates and single cells could hardly be observed at any growth stage. Cells of strain LSv54^T (Fig. 1d) often appeared in pairs. In exponentially growing cultures frequently one or two shorter cells between two long cells could be observed. Cells of LSv514^T (Fig. 1e) were short rods often growing in clumps containing up to several hundred cells. In old cultures of strains ASv26^T, LSv21^T, PSv29^T and LSv54^T some cells were motile. All isolated strains stained Gram-negative.

Growth conditions. All strains had pH optima in the neutral range (Table 1). Marine sodium concentrations supported optimum growth of ASv26^T, LSv21^T, PSv29^T, and LSv514^T but the lower range of optimal sodium concentrations differed slightly (Table 1). Although strain LSv54^T was enriched and isolated on medium with a marine salt concentration of 2.5% NaCl and 1.1% $MgCl_2 \cdot 6H_2O$ it had a remarkably lower salt optimum of 1% NaCl and 0.034 to 0.7% $MgCl_2 \cdot 6H_2O$. Marine magnesium concentrations supported optimum growth of strains ASv26^T, LSv21^T, PSv29^T and LSv514^T. All strains grew well at a temperature of -1.8°C but optimum growth temperatures were up to 20°C higher (Table 1). LSv21^T and LSv514^T had highest growth rates at about 18°C

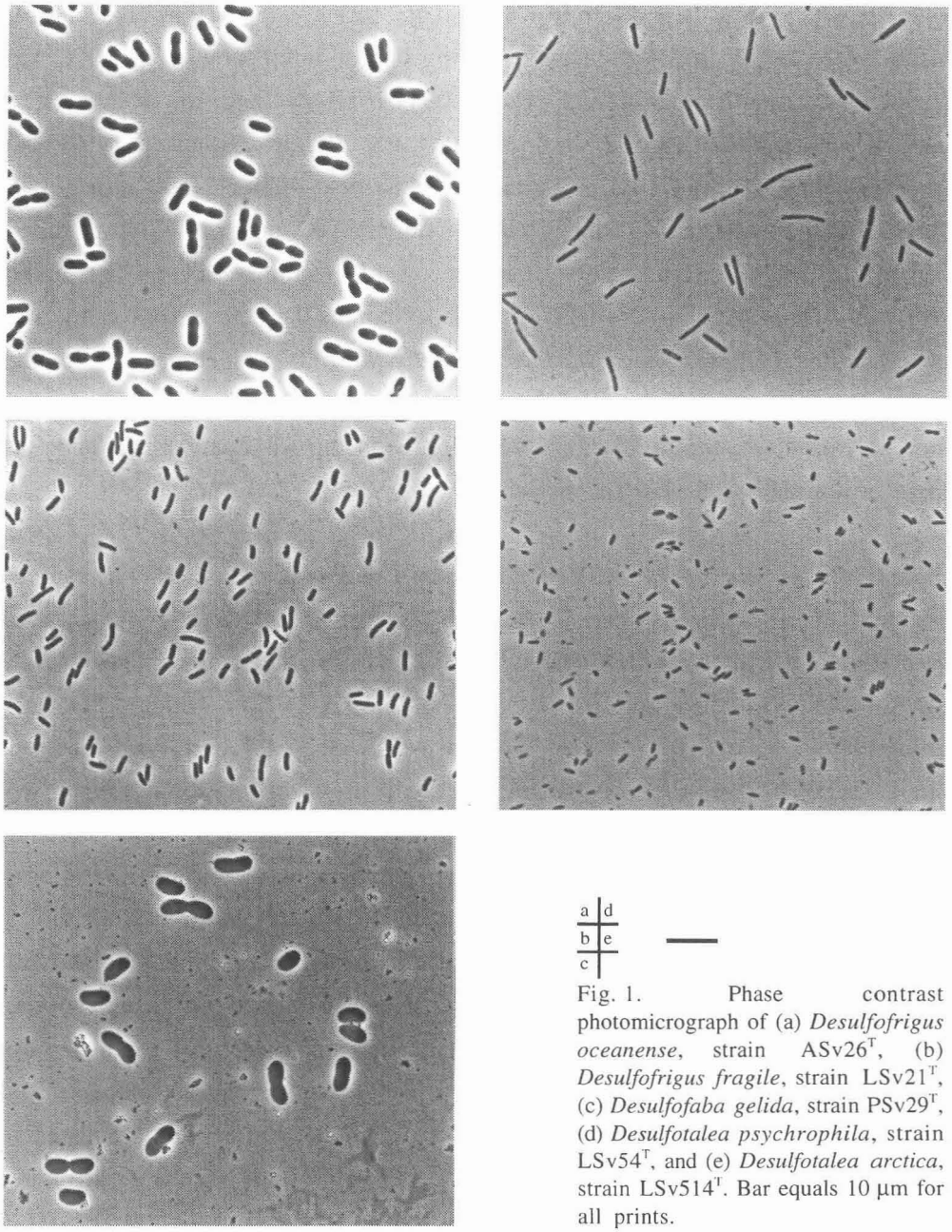

Fig. 1. Phase contrast photomicrograph of (a) *Desulfofrigus oceanense*, strain ASv26^T, (b) *Desulfofrigus fragile*, strain LSv21^T, (c) *Desulfofaba gelida*, strain PSv29^T, (d) *Desulfotalea psychrophila*, strain LSv54^T, and (e) *Desulfotalea arctica*, strain LSv514^T. Bar equals 10 μm for all prints.

with growth rates, $\mu = 0.036\ h^{-1}$ and $\mu = 0.021\ h^{-1}$ ($t_d = 19$ h and 33 h), respectively, when grown on lactate. A lower optimum temperature of 10°C was characteristic for strains ASv26^T ($\mu = 0.0041\ h^{-1}$, $t_d = 169$ h on acetate) and LSv54^T ($\mu = 0.026\ h^{-1}$, $t_d = 27$ h on lactate). PSv29^T grew fastest at 7°C with $\mu = 0.0048\ h^{-1}$ ($t_d = 144$ h on propionate). At the next higher temperature tested (10°C) this isolate could only reduce sulfate but not grow. A detailed description of the temperature response of the strains will be presented elsewhere (Knoblauch *et al.*, 1999a). Added vitamins were not required by any of the strains. Cultures were transferred usually with 10% inoculum since some of the strains exhibited a very long and unpredictable lag phase if smaller inocula were used. Strain LSv21^T lysed rapidly in the stationary phase and was therefore transferred every second week.

Substrate spectra. Strain ASv26^T grew on a wide variety of carbon sources (Table 1) and oxidized fatty acids such as formate, acetate, butyrate, and valerate completely to CO_2. Although LSv21^T was isolated on lactate, it grew also on longer-chain saturated fatty acids (C6, C10, C16) which were only incompletely oxidized to acetate. Fast growth was also found with different alcohols. PSv29^T was the only strain, that could grow on propionate which was incompletely oxidized to acetate. Growth was also possible on alcohols and dicarboxylic acids. The substrate spectrum of strains LSv514^T and LSv54^T was similar but distinct from the other strains LSv21^T, ASv26^T and PSv29^T (Table 1). With the exception of formate, no straight chain fatty acids were oxidized but both strains grew fast on hydrogen when acetate was added as carbon source. Lactate was incompletely oxidized to acetate. In comparison to LSv514^T, strain LSv54^T had a wider substrate spectrum, growing also on various alcohols and amino acids. The electron acceptors used by the different strains are listed in Table 1. Besides sulfate, strains ASv26^T, PSv29^T, and LSv54^T used also

Table 1. Some characteristics of new psychrophilic sulfate-reducing bacteria

Characteristic	ASv26^T	LSv21^T	PSv29^T	LSv54^T	LSv514^T
cell size (μm)					
width	2.1	0.8	3.1	0.6	0.7
length	4.2-6.1	3.2-4.2	5.4 -6.2	4.5-7.4	1.6-2.7
pH optimum	7.0-7.5	7.0-7.4	7.1-7.6	7.3-7.6	7.2-7.9
salt requirement (%)					
NaCl optimum	1.5-2.5	1.0-2.5	1.4-2.5	1.0	1.9-2.5
$MgCl_2 \cdot 6H_2O$ optimum	0.003-2.0	0.3-2.0	0.015-2.5	0.03-0.7	0.3-1.4
temperature optimum (T_{opt})/range (°C)	10 / -1.8-16	18 / -1.8-27	7 / -1.8-10	10 / -1.8-19	18 / -1.8-26
growth rate (h^{-1})/doubling time (h) at T_{opt}	0.0041/169	0.036/19	0.0048/144	0.026/27	0.021/33
electron donors*†					
formate (20)	++	+	+	++	++
acetate (10)	++	-	-	-	-
propionate	-	-	++	-	-
butyrate (5)	++	+	+	-	-
valerate (5)	+	-	-	-	-
caproate (3)	-	++	-	-	-
caprate (2)	-	++	-	-	-
palmitate (2)	-	+-	-	-	-
lactate (10)	++	++	++	++	++
pyruvate (10)	+	++	+	++‡	++
malate (10)	++	++	+	+-	-
succinate (10)	-	-	+	-	-
fumarate (10)	-	+	+	++	-
ethanol (10)	++	++	++	++	++
propanol (10)	++	++	++	++	-
butanol (10)	++	++	++	++	-
glycerol (10)	+-	++	+-	-	+
glycine (10)	+-	-	+-	+	-
alanine (10)	-	+	+	+	-
serine (10)	+-	+	-	+	+-
H_2/CO_2 + acetate (2)	+-	-	-	++	++

*Numbers in parenthesis represent the concentrations of components in mM
†sulfate (28 mM) was used as electron acceptor, ++ = substrate oxidized after 6 weeks, + = substrate oxidized after 4 months, +- = substrate oxidized after 8 months or more, - = substrate not oxidized; substrates tested but not oxidized: formate (autotrophic), isovalerate (5), methanol (10), glutarate (10), betaine (10), cholin chloride (10), L-proline (10), D-sorbitol (5), D-mannitol (5), benzoate (1), nicotinate (1) H_2 (autotrophic), glucose (5), fructose (5).
‡no sulfide produced, only fermentative growth

Table 1. Some characteristics of new psychrophilic sulfate-reducing bacteria (continued)

Characteristic	ASv26^T	LSv21^T	PSv29^T	LSv54^T	LSv514^T
electron acceptors*[†]					
sulfate (28)	+	+	+	+	+
thiosulfate (10)	+	-	+	+	-
sulfite (2)	+	-	+	+	-
sulfur	-	-	-	-	-[§]
Fe(III)-citrate (30)	+	+	+	+	+
Fe(III)-oxyhydroxide	-	-	-	-	-[¶]
fermentable compounds*					
pyruvate (10)	+	+	+	+	+
malate (10)	+	+	-	-	-
lactate (20)	+	-	-	-	-
fumarate (10)	-	-	+	+	-
polar lipids[Ω] and	PE, PG	PE, PG	PE, PG	PE, PG, DPG	PE, PG, DPG
major menaquinones	MK-9	MK-9	MK-8	MK-6H$_2$	MK-6
G+C molar content (%)	52.8	52.1	52.5	46.8	41.8

*Numbers in parenthesis represent the concentrations of components in mM
[§]reduction of elemental sulfur up to a concentration of 3 mM but no growth
[¶]4 mM Fe(III) reduced after 1 year
[†]nitrate and nitrite were not reduced by any of the strains
[Ω]PE = phosphatidyl ethanolamine, PG = phosphatidyl glycerol, DPG = diphosphatidyl glycerol

thiosulfate and sulfite as electron acceptor. ASv26^T, LSv21^T, LSv54^T, and LSv514^T grew with Fe(III), added as ferric-citrate, as an electron acceptor. LSv514^T also reduced Fe(III)-oxyhydroxide and sulfur very slowly but could not grow on these electron acceptors. All isolated strains fermented pyruvate but none disproportionated thiosulfate or elemental sulfur.

Pigments, polar lipids, and respiratory quinones. Desulfoviridin was not detected in any of the strains. The major polar lipids of all strains were phosphatidylethanolamine and phosphatidylglycerol (Table 1), in strains LSv54^T and LSv514^T additionally diphosphatidylglycerol was present in lower amounts. As indicated in Table 1 all strains contained menaquinones (MK). In strains ASv26^T and LSv21^T only MK-9 was present, which is uncommon for sulfate-reducing bacteria and has only been found in *Desulfonema magnum*

(Collins and Widdel, 1986). PSv29^T contained MK-8 as the sole menaquinone, which is also rare in sulfate-reducing bacteria but is characteristic for sulfur-reducing *Desulfuromonas* strains (Collins and Widdel, 1986). In LSv54^T, the major menaquinone was MK-6H_2 but also traces (≈1%) of MK-5H_2 were present. LSv514^T only contained MK-6.

Cellular fatty acids. The cellular fatty acids of the different strains are presented in Table 2. The new isolates contained none of the fatty acids that are characteristic for known sulfate-reducing bacteria, namely the branched fatty acids for *Desulfovibrio* species (Vainshtein *et al.*, 1992), the 10me 16:0 for *Desulfobacter* species (Dowling *et al.*, 1986), or the 17:1*c*9 for *Desulfobulbus* species (Taylor and Parkes, 1983). The two strains ASv26^T and LSv21^T had a similar fatty acid pattern dominated by 16:1*c*9, 18:1*c*11 and 16:0. Only even numbered fatty acids were present, an indication for the use of acetyl-CoA as precursor for chain elongation during the synthesis of fatty acids (Taylor and Parkes, 1983). Both strains contained a fatty acid (4.0 and 5.6% respectively) with an equivalent chain length of 15.49 that could not be identified. A completely different pattern was found in strain PSv29^T which was dominated by the 15:0 fatty acids. This acid was also found with 23% in *Desulfobulbus* species grown on propionate (Taylor and Parkes, 1983), which was also the carbon source of PSv29^T. The second most abundant fatty acid, 15:1*c*9, is uncommon in sulfate-reducing bacteria and was until now found only in low amounts in a *Desulfobacter* spp. grown on a mixture of fatty acids (Dowling *et al.*, 1986). In strain PSv29^T, 80% of the identified fatty acids were odd numbered, an indication for propionyl-CoA as precursor for chain elongation. The same result was also found in different *Desulfobulbus* species (Taylor and Parkes, 1983). Two fatty acids with an equivalent chain length of 14.80 (5.5%) and 19.47 (7.4%) could not be identified. Strains LSv54^T and LSv514^T

Table 2. Fatty acid composition of psychrophilic sulfate-reducing bacteria

fatty acid	ASv26^T	LSv21^T	PSv29^T	LSv54^T	LSv514^T	*"D'rhopalus vacuolatus"* ltk10^T
10:0	-	2.5	-	-	-	-
12:0	-	0.6	-	-	-	-
13:0	-	-	2.0	-	-	-
*14:0	6.7	5.0	4.9	1.4	-	-
*14:1*c*9	0.9	-	0.5	1.1	1.5	-
15:0	-	-	**38.1**	0.4	-	7.7
*15:0 3OH	-	-	0.3	-	-	2.7
15:1*c*9	-	-	**24.7**	1.7	-	**11.7**
*16:0 alde	-	-	-	-	-	2.3
16:0	9.3	**21.7**	5.4	6.5	8.3	1.7
16:0 3OH	-	-	-	-	-	0.6
16:1*c*7	0.7	3.0	0.5	1.5	3.6	-
16:1*c*9	**43.7**	**30.6**	6.5	**55.0**	**53.9**	2.9
16:1*c*11	2.2	0.5	1.5	**25.5**	**29.4**	6.3
17:0	-	-	1.7	-	-	-
17:1*c*11	-	-	4.4	2.4	-	**60.4**
18:0	0.6	0.7	0.3	-	-	0.3
18:0 3OH	-	-	0.3	-	-	-
18:0 alde	-	-	-	-	-	1.6
18:1*c*9	2.8	8.6	-	-	-	-
18:1*c*11	**23.0**	**18.5**	-	1.5	1.5	-
18:1*c*13	0.4	-	-	0.7	1.0	-
20:1*c*13	-	0.3	-	-	-	-

*abbreviations exemplified by: 14:0, tetradecanoic; 14:1*c*9, 9-tetradecenoic, double bond cis-standing; 15:0 3OH, 3-hydroxy-heptadecanoic; 16:0 alde, hexadecanal; major fatty acids are printed in bold

exhibited a very similar fatty acid pattern which was clearly different from the other new strains. The dominant fatty acids were the monounsaturated 16:1*c*9 and 16:1*c*11 comprising more than 80% of the total identified fatty acids. Like in ASv26^T and LSv21^T, the strains have preferentially even chain fatty acids. "*Desulforhopalus vacuolatus*" strain ltk10^T was included in our study since it was so far the only known moderately psychrophilic sulfate-reducing bacterium. It is most closely related to strains LSv54^T and LSv514^T. The fatty acid pattern of "*Desulforhopalus vacuolatus*" was clearly dominated by the

17:1*c*11 fatty acid. Although "*Desulforhopalus vacuolatus*" was grown on lactate, propionyl-CoA was used as precursor for chain elongation, since odd numbered fatty acids dominated with 87% of the identified acids.

G+C content of genomic DNA. The molar G+C content of strains ASv26^T, LSv21^T and PSv29^T were very similar, 52-53% (Table 1). A lower content was found in LSv54^T (47%) and LSv514^T (42%).

Phylogenetic affiliation. The 16S rDNA sequences showed that all isolates belonged to the δ subclass of *Proteobacteria*. Although they share a common characteristic in being psychrophilic, they do not form a cluster within the δ subclass but are distributed between groups of mesophilic sulfate-reducing bacteria (Fig. 2). All isolates had at least 9% evolutionary distance from 16S rDNA sequences of known mesophilic sulfate-reducing bacteria. Based on their 16S rDNA sequence, strains ASv26^T and LSv21^T are closely related to each other with an evolutionary distance of 3.2%. We regard them as different species since they showed distinct physiological differences with different substrate spectra and with ASv26^T being a complete oxidizer and LSv21^T being an incomplete oxidizer. The closest relative to both strains was PSv29^T with an evolutionary distance of 10.4 and 10.5%, respectively, followed by *Desulfosarcina variabilis* with a distance of 11.6% to ASv26^T and 12.2% to LSv21^T. PSv29^T was not closely related to any known strain but shares the highest 16S rDNA similarity with *Desulfosarcina variablis* (9.5% evolutionary distance). Strains LSv54^T and LSv514^T were closely related having 3.3% evolutionary distance. Since their physiological and chemotaxonomic

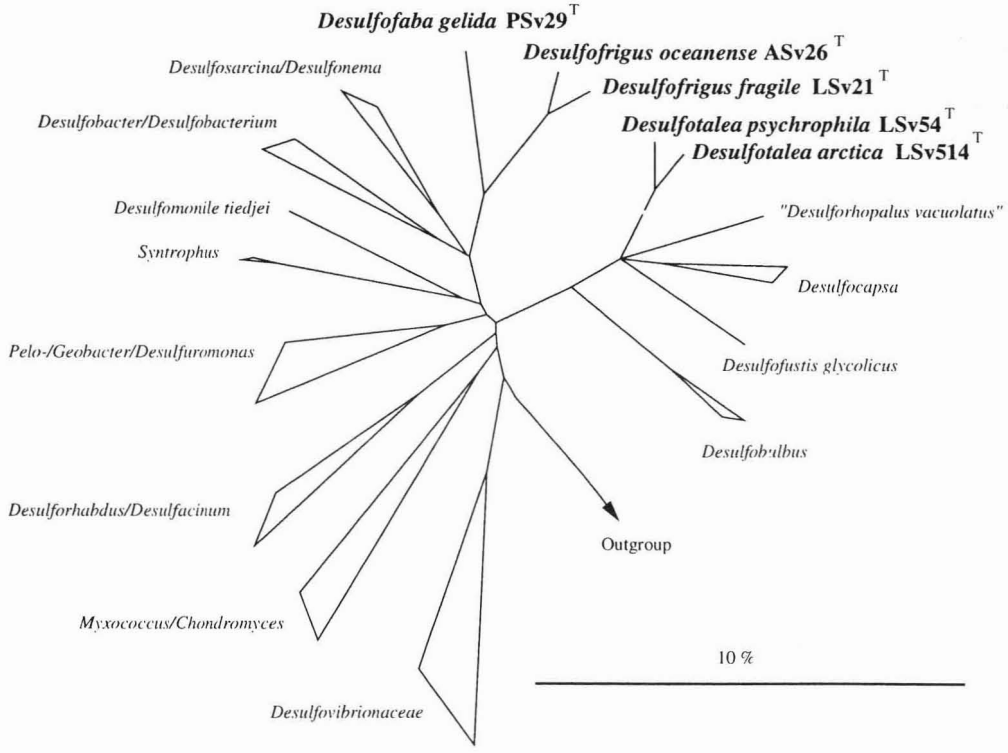

Fig. 2. Distance tree based on 16S rRNA sequences. The tree shows the δ subclass of Proteobacteria and was constructed by Neighbour-joining using a 50 % conservation filter. It depicts the phylogenetic distances using Jukes-Cantor correction with the scalebar representing 10% estimated sequence divergence. Areas of interest where the branching order changed when different treeing methods were applied are shown as multifurcation. The arrow indicates the position of the outgroup (a selection of bacterial 16S rDNA sequences from a wide range of phyla). In addition to the Svalbard-isolates, which are shown here in bold, 37 known species from the δ subclass of Proteobacteria were selected to reconstruct the tree. These reference species are shown here as groups for a better overview.

properties were quite similar (Table 1) we performed DNA-DNA hybridization to establish whether they belonged to the same species. The relative binding ratio for DNA-DNA hybridization of these two isolates was below 20%, therefore well below the threshold value of 70% accepted for the distinction of

different species (Wayne *et al.*, 1987). Based on these results, strains LSv54^T and LSv514^T can be regarded as two different species. The closest relatives of both strains were *Desulfofustis glycolicus* and "*Desulforhopalus vacuolatus*" with an evolutionary distance of 9.0-9.3%.

DISCUSSION

Ecology. The psychrophilic isolates from permanently cold Arctic sediments are the first known sulfate reducers that are able to grow below 0°C. The existence of low-temperature adapted sulfate reducers was evident, since previous studies demonstrated that rates of sulfate reduction in polar sediments were comparable to those from temperate environments (Sagemann *et al.*, 1998; Nedwell *et al.*, 1993). The new strains LSv54^T and LSv514^T grew fastest on lactate, pyruvate, alcohols and hydrogen, which are the characteristic carbon and energy substrates of *Desulfovibrio* species. In the present study, no organisms morphologically resembling *Desulfovibrio* strains were detected in any enrichment incubated between 0 and 10°C and no *Desulfovibrio* strain was isolated at low temperatures. These results are unusual, since *Desulfovibrio* strains are generally dominant in lactate containing enrichment cultures (Postgate, 1984). The major difference between previous enrichments and ours was the temperature used for incubation, 28-36°C versus 0-10°C, indicating that growth temperature might affect the outcome of competition between different groups of sulfate reducers. This was also supported by the results of Isaksen and Teske (1996) who enriched sulfate reducers with lactate from a temperate estuary at 10°C. They isolated the moderate psychrophilic strain ltk10^T, which they described as the type strain of a new genus "*Desulforhopalus vacuolatus*". However, it might also be that *Desulfovibrio* species are not abundant in the investigated habitat, and that their ecological niche is taken by species related to LSv54^T and LSv514^T. Strains LSv21^T and PSv29^T oxidize

various fatty acids such as propionate, butyrate, caproate, caprate and even palmitate incompletely to acetate which can be further oxidized by strain ASv26^T to CO_2. Besides lactate and hydrogen, volatile fatty acids are the most important end products of fermentation and are the major organic substrates for sulfate reducers in temperate environments (Sørensen *et al.*, 1981; Christensen, 1984; Parkes *et al.*, 1989). The fact that all these compounds were also oxidized at sub-zero temperatures by the new psychrophilic isolates is a further indication that these bacteria occupy the same ecological niche in cold sediments as the mesophiles in temperate sediments.

Chemotaxonomy. According to their cellular fatty acid pattern the new strains can be assigned to three distinct groups. The major fatty acids of both ASv26^T and LSv21^T were the 16:1*c*9, 18:1*c*11 and the 16:0 acid (Table 2). This combination is unique among the known sulfate reducers and supports the assignment of these two isolates to a new genus. PSv29^T, the closest relative to ASv26^T and LSv21^T, had a completely different pattern with the 15:0 and the 15:1*c*9 fatty acids dominating. Both fatty acids were absent in *Desulfosarcina variabilis* (Kohring *et al.*, 1994), which is phylogenetically closest related to PSv29^T. The third group comprised strain LSv54^T and LSv514^T. Both have a very similar fatty acid pattern dominated by 16:1*c*9 and 16:1*c*11, a combination which to our knowledge has not been found in any other sulfate reducer. "*Desulforhopalus vacuolatus*" strain ltk10^T was included in our study since it is moderately psychrophilic and most closely related to strains LSv54^T and LSv514^T. Its fatty acid pattern was dominated by the 17:1*c*11 acid (Table 2) but it contained also 12% of the 15:1*c*9 acid. Therefore, in the euclidian distance tree (data not shown) ltk10^T was closer related to PSv29^T than to LSv54^T and LSv514^T. These results cannot be due to differences in the growth conditions, because ltk10^T, LSv54^T and LSv514^T were all cultivated with lactate on the same medium at 4°C. The percentage of unsaturated fatty acids in strains LSv54^T and

LSv514^T of 89 and 91% (Table 2) is remarkably high. Among the mesophilic sulfate-reducing bacteria the highest content of unsaturated fatty acids was 62% in *Desulfovibrio africanus* (Vainshtein *et al.*, 1992). The high degree of unsaturated fatty acids in the new strains can be an adaptation to low temperature. Unsaturated fatty acids lower the gel-liquid-crystalline phase transition temperature of membranes (Russell, 1990), thereby maintaining the necessary fluidity at low growth temperatures. High concentrations of unsaturated fatty acids were found also in ASv26^T and LSv21^T (Table 2) but not in PSv29^T. Another way to increase membrane fluidity is to decrease the fatty acyl chain length (Bhakoo and Herbert, 1979). This is the case in PSv29^T which contains more than 70% fatty acids with a chain length of 15 carbon atoms or less. It is not known if sulfate-reducing bacteria alter their membrane composition when grown at different temperatures.

The menaquinone analyses showed similar relationships between the new isolates as do the fatty acid profiles. ASv26^T and LSv21^T contained only MK-9, which was not fond in any of the other new isolates and out of 45 sulfate-reducing bacteria strains investigated by Collins and Widdel (1986) it only occurred in *Desulfonema magnum*. The sole menaquinone in PSv29^T was MK-8, which is absent in its closest relative, *Desulfosarcina variabilis* (Collins and Widdel, 1986). Menaquinones with six isoprenoid subunits were dominant in strains LSv54^T and LSv514^T (Table 1). These menaquinones are characteristic for most *Desulfovibrio* strains (Collins and Widdel, 1986). Unfortunately, menaquinone data are not available for their closest relatives, *Desulfofustis glycolicus* and "*Desulforhopalus vacuolatus*". The chemotaxonomic data were in good agreement with the 16S rRNA sequence data, which showed the same affiliation between the different isolates (Fig. 2). More conflicting results arose from the substrate spectra of strains ASv26^T and LSv21^T. Although most closely related to ASv26^T (evolutionary distance 3.2%) strain LSv21^T oxidized fatty acids only incompletely to acetate. Complete or

incomplete substrate oxidation was traditionally used as a criterion to distinguish genera of sulfate-reducing bacteria (Devereux *et al.*, 1989; Holt *et al.*, 1994). These distinctions have been supported by phylogenetic data. The lowest evolutionary distance between a completely oxidizing species (*Desulfococcus multivorans*) and an incompletely oxidizing sulfate-reducing bacteria (*Desulfovibrio sapovorans*) was 11%. Our results demonstrate that complete substrate oxidation to CO_2 is not always a phylogenetically deep branching property. Chemotaxonomic parameters such as fatty acid pattern and menaquinone content of the different isolates were in closer agreement with the phylogenetic data than the substrate spectra.

Strains LSv54^T and LSv514^T were phylogenetically closest related to *Desulfofustis glycolicus* and "*Desulforhopalus vacuolatus*". The new strains shared only few general characteristics with the genus *Desulfofustis*, e.g. the absence of desulfoviridin and the incomplete oxidation of lactate. The most conspicuous feature, which the new strains shared with "*Desulforhopalus vacuolatus*", is psychrophily. On the other hand, the new strains could be easily differentiated from "*Desulforhopalus vacuolatus*" by the absence of gas vacuoles, by their inability to grow on propionate, and by their completely different cellular fatty acid pattern.

Taxonomic affiliation. The phylogenetically closest relative to ASv26^T and LSv21^T was PSv29^T with evolutionary distances of 10.4 and 10.5%, respectively. Due to this phylogenetic distance, as well as dissimilarities in physiology, the fatty acid pattern, and menaquinone content we propose for ASv26^T and LSv21^T the establishment of a new genus, *Desulfofrigus*. On the basis of an evolutionary distance of 3.2%, distinct morphologies, temperature responses of growth, and substrate spectra we classify ASv26^T and LSv21^T as two species of the genus *Desulfofrigus*. ASv26^T is the type strain of the type species *Desulfofrigus oceanense* and LSv21^T is the type strain of *Desulfofrigus fragile*.

Due to the evolutionary distance of 9.5% between PSv29^T and *Desulfosarcina variabilis* and the differences in their physiology and chemotaxonomic properties, we propose the establishment of the new genus *Desulfofaba* with PSv29^T as type strain of the type species *Desulfofaba gelida*.

Considering the phylogenetic distance between strains LSv54^T, LSv514^T and "*Desulforhopalus vacuolatus*" as well as their physiological, morphological and chemotaxonomic differences, we place strain LSv54^T and LSv514^T in a new genus *Desulfotalea*. Since both strains have distinct physiological properties and a DNA-DNA similarity of less than 70% we define two new species of the genus *Desulfotalea*, with LSv54^T as type strain of the type species *Desulfotalea psychrophila* and with LSv514^T as type strain of *Desulfotalea arctica*.

Description of *Desulfofrigus* gen. nov.

De.sul.fo.fri´gus. L. prefix *de*, off; L. n. *sulfur*, sulfur; L. neut. n. *frigus*, cold; M. L. neut. n. *Desulfofrigus*, sulfate reducer living in the cold. Members are Gram-negative obligately anaerobic bacteria and belong to the δ subdivision of *Proteobacteria*. Sulfate is used as terminal electron acceptor and reduced to sulfide. Fe(III) can also be used as electron acceptor. Fermentative growth on pyruvate or other carbon substrates. Chemoorganotrophic growth on fatty acids and alcohols that are either completely oxidized to CO_2 or incompletely to acetate. No chemoautotrophic growth. Major cellular fatty acids are even, mono-unsaturated and unbranched. MK-9 is the dominant menaquinone. The type species of this genus is *Desulfofrigus oceanense* strain ASv26^T. A second member of this genus is *Desulfofrigus fragile*.

Description of *Desulfofrigus oceanense* gen. nov., sp. nov.

o.ce.a.nen´se. L. adj. *oceanensis*, belonging to the ocean. Cells are 2.1 µm wide and 4.2 to 6.1 µm long. The pH optimum is between 7.0 and 7.5. They require sodium chloride and optimum growth occurs at marine salt concentrations. The temperature optimum for growth is at 10°C and they grow also at -1.8°C. They contain the polar lipids phosphatidylethanolamine and phosphatidylglycerol, and MK-9 is the sole menaquinone. Major cellular fatty acids are 16:1*c*9 and 18:1*c*11. The molar G+C content is 52.8%. As electron acceptors they use sulfate, thiosulfate, and sulfite which are reduced to sulfide. Fe(III) serves as an electron acceptor when added as Fe(III)-citrate. Elemental sulfur, Fe(III)-oxyhydroxide, nitrate and nitrite are not reduced. Formate, acetate, butyrate, valerate, lactate, pyruvate, malate, ethanol, propanol, butanol, glycerol, glycine, and serine serve as carbon substrates. Fatty acids are oxidized completely to CO_2. Growth on H_2 plus acetate (2 mM) is slow. Fermentative growth on pyruvate, malate, and lactate. Vitamins are not required for growth. Desulfoviridin is absent. Elemental sulfur and thiosulfate are not disproportionated. Their habitat is permanently cold marine sediments. Strain ASv26^T is the type strain of the species and is deposited at the DSMZ under number 12341^T. The 16S rDNA sequence was deposited at GenBank under accession number AF099064.

Description of *Desulfofrigus fragile* sp. nov.

fra´gi.le. L. adj. *fragilis*, referring to the rapid lysis of the type strain in the stationary phase. Cells are 0.8 µm wide and 3.2 to 4.2 µm long. The pH optimum is between 7.0 and 7.4. They require sodium chloride, and optimum growth occurs on marine salt concentrations. The temperature optimum for growth is 18°C and they grow also at -1.8°C. They contain the polar lipids phosphatidylethanolamine and phosphatidylglycerol, and MK-9 is the sole menaquinone. Major cellular fatty acids are 16:1*c*9, 16:0 and 18:1*c*11. The

molar G+C content is 52.1%. As electron acceptor they use sulfate which is reduced to sulfide. Elemental sulfur, sulfite, thiosulfate, nitrate, nitrite and Fe(III)-oxyhydroxide are not reduced. Fe(III) serves as electron acceptor when added as Fe(III)-citrate. Formate, butyrate, caproate, caprate, palmitate, lactate, pyruvate, malate, fumarate, ethanol, propanol, butanol, glycerol, alanine, and serine serve as carbon sources and electron donors. Fatty acids are oxidized incompletely to acetate. Fermentative growth on pyruvate and malate. Vitamins are not required for growth. Desulfoviridin is absent. Cells lyse rapidly in the stationary growth phase. Elemental sulfur and thiosulfate are not disproportionated. Their habitat is permanently cold marine sediments. Type strain LSv21^T is deposited at the DSMZ under number 12345^T. The 16S rDNA sequence was deposited at GenBank under accession number AF099065.

Description of *Desulfofaba* gen. nov.

De.sul.fo.fa´ba. L. prefix *de*, off, L. n. *sulfur*, sulfur; L. fem. n. *faba*, a bean; M. L. fem. n. *Desulfofaba*, a sulfate-reducing bean. Members are Gram-negative, obligately anaerobic bacteria belonging to the δ subdivision of *Proteobacteria*. Sulfate is used as electron acceptor and reduced to sulfide. Fermentative growth on pyruvate or other carbon substrates is possible. Major carbon sources and electron donors are fatty acids and alcohols that are oxidized incompletely to acetate. Major cellular fatty acids are odd numbered and unbranched. MK-8 is the dominant menaquinone. The only species, *Desulfofaba gelida*, strain PSv29^T is the type species of the genus.

Description of *Desulfofaba gelida* gen. nov., sp. nov.

ge´li.da, L. adj. *gelidus*, ice-cold; referring to the low temperature optimum. Cells are 3.1 µm wide and 5.4 to 6.2 µm long. The pH optimum is between 7.1 and 7.6. They require sodium chloride and magnesium chloride, and optimum growth occurs at marine salt concentrations. The temperature optimum for

growth is at 7 °C and they grow also at -1.8 °C. They contain the polar lipids phosphatidylethanolamine and phosphatidylglycerol, and MK-8 is the sole menaquinone. Major cellular fatty acids are 15:0 and 15:1*c*9. The molar G+C content is 52.5 %. As electron acceptor they use sulfate, thiosulfate and sulfite which are reduced to sulfide. Elemental sulfur, nitrate, nitrite, Fe(III)-oxyhydroxide, and Fe(III)-citrate are not reduced. Sulfur and thiosulfate are not disproportionated. Formate, propionate, butyrate, lactate, pyruvate, malate, succinate, fumarate, ethanol, propanol, butanol, glycerol, glycine, and alanine serve as carbon sources. Fatty acids are oxidized incompletely to acetate. Fermentative growth on pyruvate and fumarate. Vitamins are not required for growth. Desulfoviridin is absent. Their habitat is permanently cold marine sediments. Type strain PSv29^T is deposited at the DSMZ under number 12344^T. The 16S rDNA sequence was deposited at GenBank under accession number AF099063.

Description of genus *Desulfotalea* gen. nov.

De.sul.fo.ta´le.a. L. prefix *de*, off, L. n. *sulfur*, sulfur; L. fem. n. *talea*, a rod; M. L. fem. n. *Desulfotalea*, a sulfate-reducing rod. Members are Gram-negative obligately anaerobic bacteria belonging to the δ subdivision of *Proteobacteria*. Sulfate is used as electron acceptor and reduced to sulfide. Fermentative growth on pyruvate. Fe(III) can be used as electron acceptor. Major carbon or energy sources are lactate, alcohols and hydrogen. Cellular fatty acids comprise even numbered, mono-unsaturated acids. The dominant menaquinones have six isoprenoid units. *Desulfotalea psychrophila* strain LSv54^T is the type species of the genus. A second member of this genus is *Desulfotalea arctica*.

Description of *Desulfotalea psychrophila* gen. nov., sp. nov.

psy.chro´phi.la, Gr. adj. *psychros*, cold; *philos*, loving; ML adj. psychrophilus, cold loving. Cells are 0.6 µm wide and 4.5 to 7.4 µm long. The pH optimum is

between 7.3 and 7.6. They require sodium chloride and magnesium chloride for growth. The optimum sodium chloride concentration is 1 %. The temperature optimum for growth is at 10°C and they grow also at -1.8°C. Cells contain the polar lipids phosphatidylethanolamine, phosphatidylglycerol and diphosphatidyl glycerol. MK-$6H_2$ is the major menaquinone but traces of MK-$5H_2$ are also present. Major cellular fatty acids are 16:1*c*9 and 16:1*c*11. The molar G+C content is 46.8%. Sulfate, thiosulfate and sulfite are used as electron acceptors which are reduced to sulfide. Elemental sulfur, nitrate, nitrite, and Fe(III)-oxyhydroxide are not reduced. Growth by reduction of Fe(III), if added as Fe(III)-citrate, is possible. Elemental sulfur or thiosulfate are not disproportionated. Formate, lactate, pyruvate, malate, fumarate, ethanol, propanol, butanol, glycine, alanine, and serine serve as carbon sources. They grow on hydrogen plus acetate (2 mM). Organic substrates are oxidized incompletely to acetate. Fermentative growth on pyruvate and fumarate. Vitamins are not required. Desulfoviridin is absent. Their habitat is permanently cold marine sediments. Type strain is LSv54^T which is deposited at the DSMZ under number 12343^T. The 16S rDNA sequence was deposited at GenBank under accession number AF099062.

Description of *Desulfotalea arctica* sp. nov.

arc´ti.ca, L. adj. *arcticus*, from the Arctic, referring to the site were the type strain was isolated. Cells are 0.7 µm wide and 1.6 to 2.7 µm long. The pH optimum is between 7.2 and 7.9. Sodium chloride and magnesium chloride are required for growth. Optimum growth occurs at marine salt concentrations. The temperature optimum for growth is at 18°C and they grow also at -1.8°C. Cells contain the polar lipids phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. MK-6 is the sole menaquinone. Major cellular fatty acids are 16:1*c*9 and 16:1*c*11. The molar G+C content is 41.8%. Sulfate serves as electron acceptor and is reduced to sulfide. Thiosulfate, sulfite, nitrate, and

nitrite are not reduced. Growth by reduction of Fe(III), if added as Fe(III)-citrate. Elemental sulfur and Fe(III)-oxyhydroxide are slowly reduced without growth. Elemental sulfur and thiosulfate are not disproportionated. Formate, lactate, pyruvate, ethanol, glycerol, and serine serve as carbon sources and electron donors. Hydrogen plus acetate (2 mM) allows rapid growth. Organic substrates are oxidized incompletely to acetate. Fermentative growth on pyruvate. Vitamins are not required for growth. Desulfoviridin is absent. Their habitat is permanently cold marine sediments. Type strain is LSv514^T which is deposited at the DSMZ under number 12342^T. The 16S rDNA sequence was deposited at GenBank under accession number AF099061.

2.4.

Effect of temperature on sulfate reduction, growth rate, and growth yield in five psychrophilic sulfate-reducing bacteria from Arctic sediments

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Five psychrophilic sulfate-reducing bacteria (strains ASv26, LSv21, PSv29, LSv54 and LSv514) isolated from Arctic sediments were examined for their adaptation to permanently low temperatures. All strains grew at -1.8 °C, the freezing point of seawater, but their optimum temperature for growth (T_{opt}), were 7 °C (PSv29), 10 °C (ASv26, LSv54) and 18 °C (LSv21, LSv514). Although T_{opt} was considerably above the *in situ* temperatures of their habitats (-1.7 and 2.6 °C), relative growth rates were still high at 0 °C, accounting for 25-41 % of those at T_{opt}. Short-term incubations of exponentially growing cultures showed that the highest sulfate reduction rates occurred 2-9 °C above T_{opt}. In contrast to growth and sulfate reduction rates, growth yields of strains ASv26, LSv54 and PSv29 were almost constant between -1.8 °C and T_{opt}. For strains LSv21 and LSv514, however, growth yields were highest at the lowest temperatures, around 0 °C. The results indicate that psychrophilic sulfate-reducing bacteria are specially adapted to permanently low temperatures by high relative growth rates and high growth yields at *in situ* conditions.

INTRODUCTION

More than 90% of the world ocean floor has temperatures below 4°C (Levitus and Boyer, 1994), hence benthic organisms must be active and grow in the cold. Temperature adaptation of microorganisms is commonly described by their optimum temperature (T_{opt}), which is the temperature at which they grow fastest. However, often their metabolical activity rather than their growth is measured. Communities in cold sediments may appear poorly adapted to their environmental temperature since their highest rate of growth or metabolism is found far above the *in situ* temperature (e.g. Nedwell, 1989; Kirchman *et al.*, 1993; Arnosti *et al.*, 1998; Sagemann *et al.*, 1998). However, the successful adaptation of bacteria to low temperatures appears from their competitiveness under *in situ* conditions rather than from the temperature at which potential growth rates are highest. Furthermore, in addition to growth rates, physiological characters such as substrate affinity (Westermann *et al.*, 1989) or growth yield (Herbert and Bell, 1977) are also temperature dependent and affect the outcome of competition among different organisms (Gottschal, 1986; and references therein).

Up to 50% of the organic carbon in marine sediments is mineralized to CO_2 by sulfate-reducing bacteria (SRB) (Jørgensen, 1982) which are active over a wide range of temperatures from below 0 to above 100°C (Jørgensen *et al.*, 1992; Nedwell *et al.*, 1993; Sagemann *et al.*, 1998). Comparable sulfate reduction rates have been found in temperate and polar sediments (Jørgensen *et al.*, 1990; Nedwell *et al.*, 1993; Thamdrup *et al.*, 1994; Sagemann *et al.*, 1998). Despite the importance of cold sediments, studies of the temperature adaptation of sulfate reduction in these habitats are scarce and the few studies conducted to date suggest the predominance of a mesophilic population with a temperature optimum between 20 and 30°C (Nedwell, 1989; Isaksen and Jørgensen, 1996; Sagemann *et al.*, 1998). All of these sediment studies are based on sulfate

reduction measurements, since growth of SRB could not be measured in the environmental samples (Gilmour *et al.*, 1990; Winding, 1992). Isaksen and Jørgensen (1996) showed that a moderate psychrophilic SRB had highest sulfate reduction rates at 28°C although the T_{opt} was ten degrees lower, at 18°C. This suggests that a mesophilic response of sulfate reduction in sediments might still be assigned to a community with a psychrophilic growth potential.

In the present study we determined temperature curves of growth, sulfate reduction, and growth yield in pure cultures of five different psychrophilic SRB that were isolated from two permanently cold (-1.7 and 2.6°C, respectively) marine sediments. We distinguish two groups of low-temperature adapted SRB according to their optimum temperature of growth. Moderate psychrophiles (psychrotrophs according to Morita, 1975) have a T_{opt} above 15°C but still grow at or below 5°C, and obligate psychrophiles (psychrophiles according to Morita, 1975) with a T_{opt} below 15°C and growth at or below 0°C. The terms T_{min} and T_{max} are used to indicate the lowest and highest temperature at which the organisms were able to grow. The aim of the present study was to further elucidate how and to what extent psychrophilic sulfate-reducing bacteria are adapted to their permanently cold environment. The presented data indicate that low-temperature adapted SRB from Arctic sediments have a physiological competitive advantage under *in situ* conditions.

RESULTS

All strains grew at the freezing point of seawater, -1.8°C, but temperature optima were much above the *in situ* temperature of Arctic sediments and varied between 7 and 18°C (Table 1). On the basis of T_{opt}, strains ASv26, PSv29 and LSv54 were classified as obligate psychrophiles, strains LSv21 and LSv514 as moderate psychrophiles. However, growth rates at environmental temperatures

Table 1. Temperature characteristics of psychrophilic isolates; tentative names (Knoblauch *et al.*, 1999b) are given in parenthesis.

strain	T_{opt} (°C)	Relative growth rate at 0 °C vs. Topt (%)	$^{*}E_a$ (± SD)	T-range used for calculation of $^{*}E_a$ (°C)
ASv26 (*Desulfofrigus oceanense*)	10	24	59.0 ± 3.3	-2 - 17
LSv21 (*Desulfofrigus fragile*)	18	31	58.2 ± 3.4	-2 - 22
PSv29 (*Desulfofaba gelida*)	7	41	†n.d.	
LSv54 (*Desulfotalea psychropila*)	10	33	54.3 ± 1.8	-4 - 12
LSv514 (*Desulfotalea arctica*)	18	29	54.0 ± 1.5	-2 - 23

$^{*}E_a$ = apparent activation energy
†n.d. = not determined

are more relevant than T_{opt} for the adaptation of psychrophilic bacteria to permanently low temperatures. Consequently, we compared growth rates at 0°C, which is close to the *in situ* temperatures of Arctic sediments, to those at T_{opt} of the isolates and found remarkably high relative rates of 24 to 41% (Table 1).

Sulfate reduction rates of all strains increased exponentially between T_{min} and T_{opt} and the Arrhenius equation provided a good fit of data in the respective temperature ranges. Whereas optimum temperatures differed substantially, apparent activation energies (E_a) varied only slightly (Table 1).

Temperature response of growth, sulfate reduction and growth yield. Strain ASv26 grew fastest at 10°C and not above 15°C (Fig. 1a). Doubling times on acetate as carbon and energy source were 7 days at T_{opt} but five weeks at the freezing point of sea water, -1.8°C. Highest sulfate reduction rates were found at 17°C (Fig. 1b), which is considerably above T_{opt} and even above the temperature range, in which strain ASv26 is able to grow. Growth yield of ASv26 was

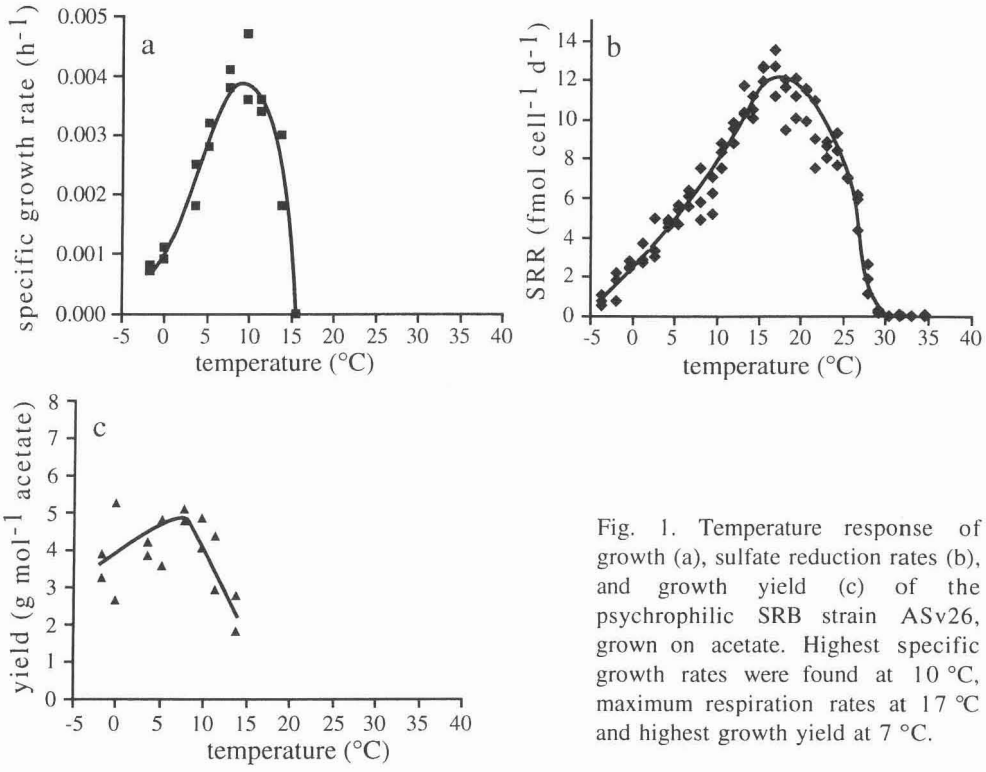

Fig. 1. Temperature response of growth (a), sulfate reduction rates (b), and growth yield (c) of the psychrophilic SRB strain ASv26, grown on acetate. Highest specific growth rates were found at 10 °C, maximum respiration rates at 17 °C and highest growth yield at 7 °C.

highest at 7°C (4.9 g dry weight mol^{-1} acetate) and decreased only slightly with decreasing temperatures to 3.5 g dry weight mol^{-1} acetate at the lowest temperature tested, -1.8°C (Fig. 1c).

Strain LSv21, phylogenetically most closely related to ASv26 (3.2% evolutionary distance, [Knoblauch *et al.*, 1999b]), was isolated on lactate. Its optimum temperature was about 18°C with a maximum specific growth rate of 0.036 h^{-1} or a doubling time of 19 hours (Fig. 2a). At -1.8°C doubling times

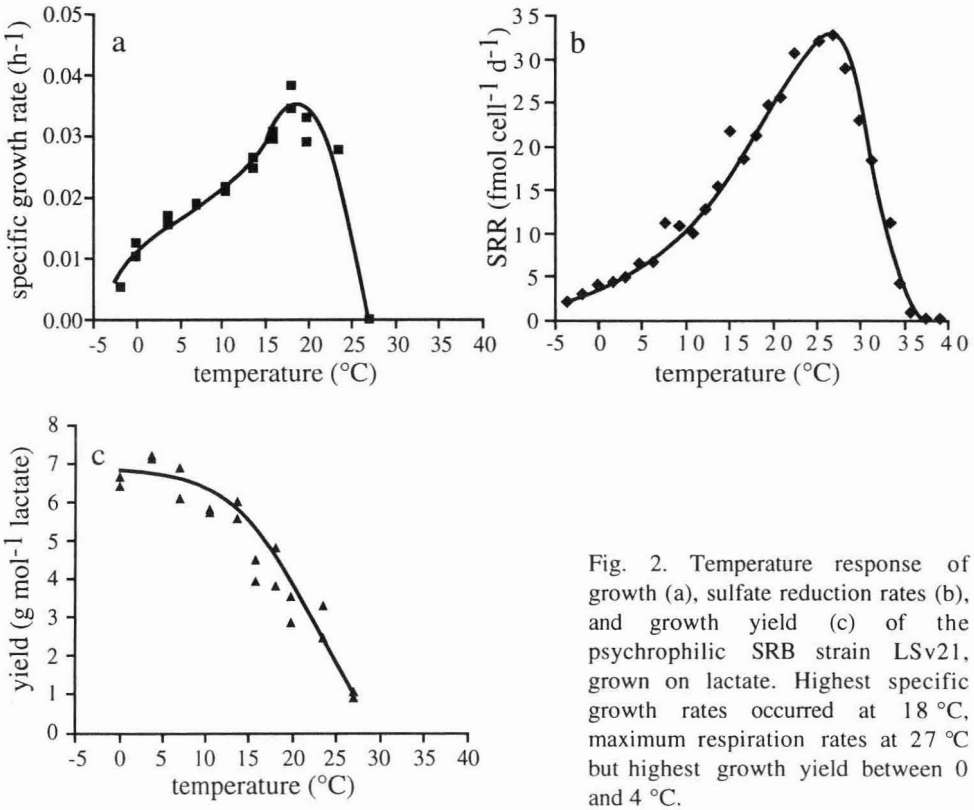

Fig. 2. Temperature response of growth (a), sulfate reduction rates (b), and growth yield (c) of the psychrophilic SRB strain LSv21, grown on lactate. Highest specific growth rates occurred at 18 °C, maximum respiration rates at 27 °C but highest growth yield between 0 and 4 °C.

increased to 5.6 days. Maximum sulfate reduction rates were measured at 27°C (Fig. 2b) which is outside its temperature range for growth. In contrast to growth and sulfate reduction, growth yield increased with decreasing temperatures and was highest (7.2 g dry weight mol^{-1} lactate) at 4°C (Fig. 2c). Between 4 and 0°C growth yield was 1.5 to 1.7 fold higher than at T_{opt}.

Strain PSv29 was the only strain which was able to oxidize propionate. It grew fastest at 7°C (Fig. 3a) thereby having the lowest temperature optimum of all

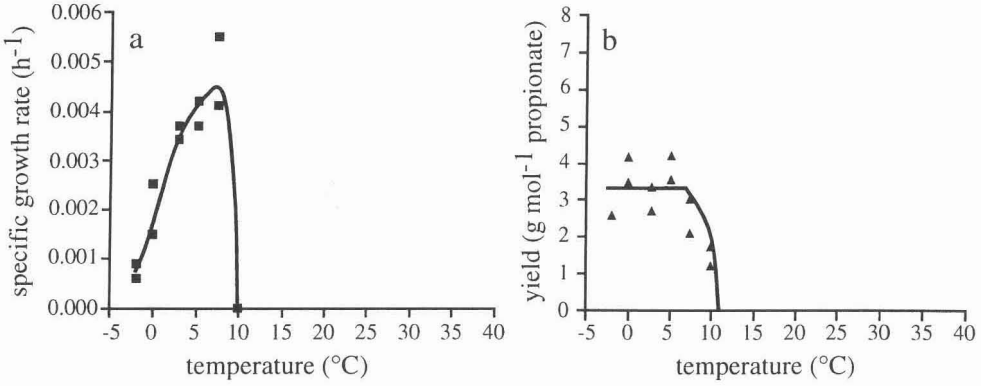

Fig. 3. Temperature response of growth (a), and growth yield (b) of the psychrophilic SRB strain PSv29, grown on propionate. Highest specific growth rates were measured at 7 °C, but growth yield was almost constant between -1.8 and 7 °C.

isolates. At 10°C it showed no substantial growth although it still produced sulfide (data not shown). Growth on propionate was slow and doubling times at T_{opt} were 6 days but increased to more than 5 weeks at -1.8°C. Growth yield was highest below T_{opt} with a maximum of 3.8 g dry weight mol propionate^{-1} at 5°C (Fig. 3b). Between -1.8 and 5°C growth yield was nearly constant with a mean value of 3.2 g dry weight mol^{-1} propionate.

Strain LSv54 and LSv514 were both isolated on lactate as carbon and energy source. They are phylogenetically closely related with an evolutionary distance of 3.3% (Knoblauch *et al.*, 1999b). The temperature optimum of LSv54 was at 10°C with a specific growth rate of 0.026 h^{-1} and a doubling time of one day (Fig. 4a). At -1.8°C, LSv54 showed highest growth rates of all isolates with doubling times of four days. Sulfate reduction was highest at 12°C (Fig. 4b) but specific sulfate reduction rates were about 10 fold lower than in strain LSv514 (Fig. 5b). These differences are most likely due to the growth

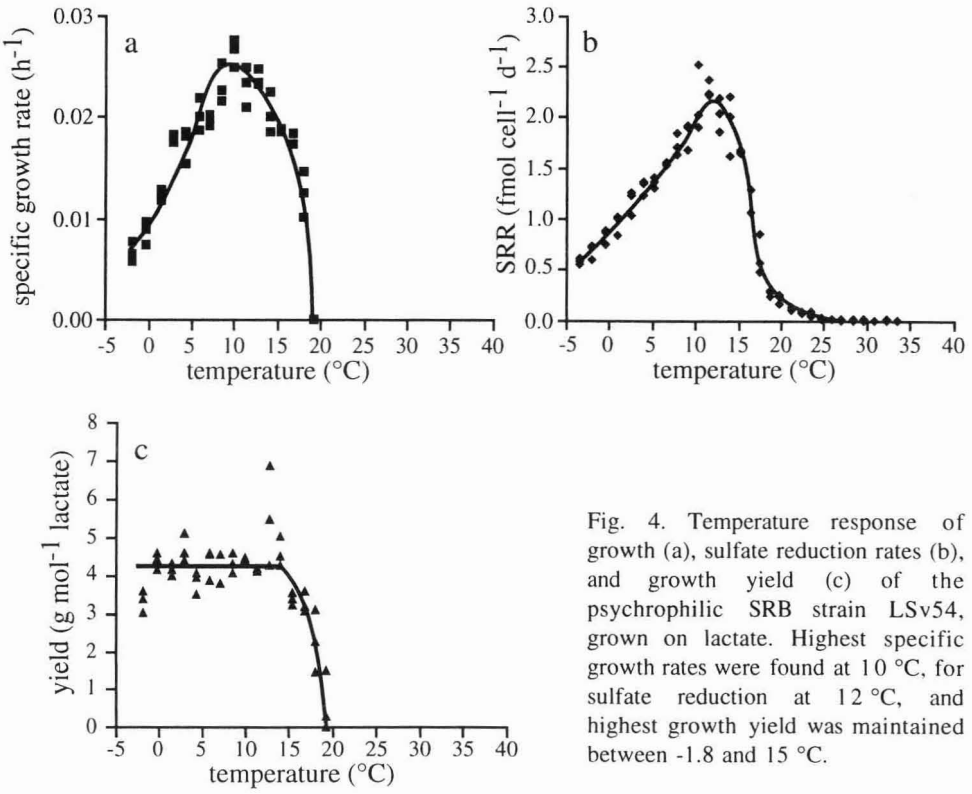

Fig. 4. Temperature response of growth (a), sulfate reduction rates (b), and growth yield (c) of the psychrophilic SRB strain LSv54, grown on lactate. Highest specific growth rates were found at 10 °C, for sulfate reduction at 12 °C, and highest growth yield was maintained between -1.8 and 15 °C.

phases of the cultures used for measurements. Sulfate reduction rates of strain LSv54 were measured in a culture grown to the late exponential growth phase whereas the LSv514 culture was in the early exponential phase. Measurements of specific sulfate reduction rates in a LSv54 culture revealed that cell-specific sulfate reduction rates are highest in the early exponential growth phase and decrease rapidly as the culture approaches the stationary growth phase (V. Brüchert, personal communication). Additional measurements with cultures of LSv54 and LSv514 in the same growth phase showed that both strains do have comparable specific activities at the *in situ* temperature of the sediment

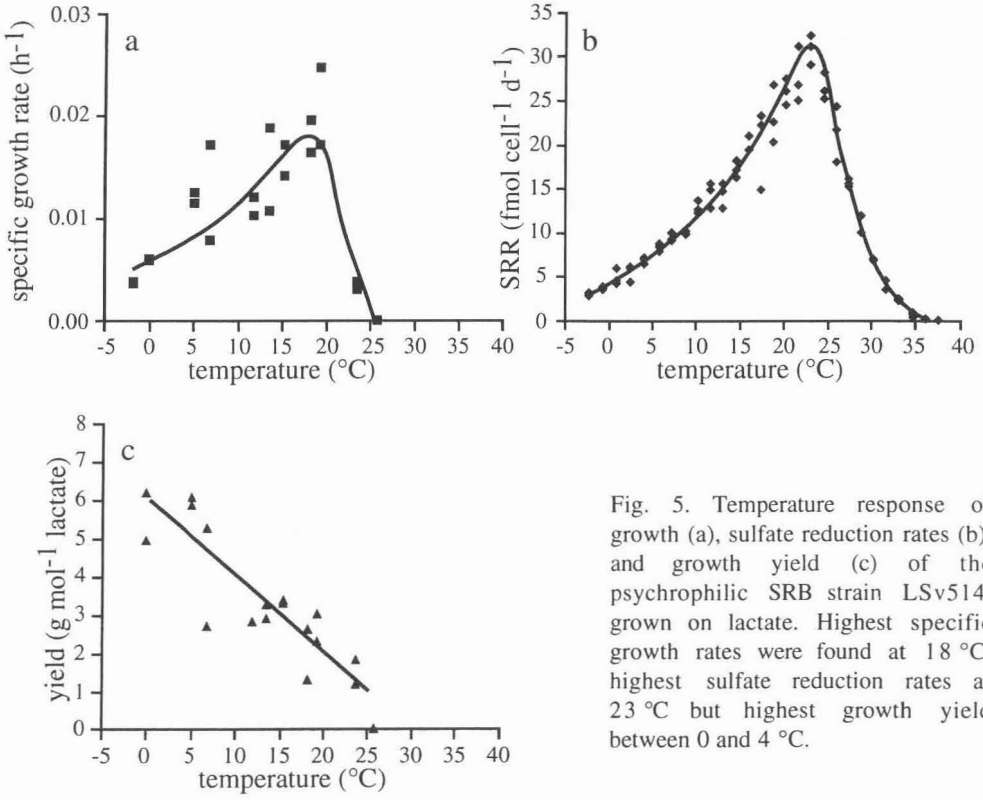

Fig. 5. Temperature response of growth (a), sulfate reduction rates (b), and growth yield (c) of the psychrophilic SRB strain LSv514, grown on lactate. Highest specific growth rates were found at 18 °C, highest sulfate reduction rates at 23 °C but highest growth yield between 0 and 4 °C.

(Knoblauch *et al.*, 1999a). The growth yield of LSv54 between T_{min} and T_{opt} was nearly constant with a mean value of 4.2 g dry weight mol^{-1} lactate (Fig. 4c).

Strain LSv514 grew fastest at 18°C with a doubling time of 33 hours (Fig. 5a). Although T_{opt} of LSv514 was 9°C higher than of LSv54, maximum growth rates of the two strains were comparable. At -1.8°C doubling time was 8 days. Sulfate reduction rates were highest at 23°C (Fig. 5b) and growth yield increased, as in LSv21, with decreasing temperatures (Fig 5c). Maximum values of 5.6-6.0 g dry weight mol^{-1} lactate were found between 4 and 0°C. Hence at temperatures

close to the *in situ* temperature of their natural environment growth yield was 2.1 times higher than at T_{opt}.

DISCUSSION

Temperature response of growth. All strains were able to grow at -1.8°C, the freezing point of seawater. Strains LSv54, PSv29, and ASv26 were obligate psychrophiles with a T_{opt} of 7 to 10°C (Table 1). These are the lowest optimal growth temperatures reported for anaerobic prokaryotes (Dyrset *et al.*, 1984; Franzmann and Rohde, 1991; Mountfort *et al.*, 1997; Franzmann *et al.*, 1997). A T_{opt} approximately ten degrees higher was found for the moderately psychrophilic strains, LSv514 and LSv21. It is a common observation that the temperature optimum of psychrophilic bacteria is well above the *in situ* temperature of their natural habitat (Delille and Perret, 1989; McMeekin and Franzmann, 1988; Franzmann *et al.*, 1997; Bowman *et al.*, 1997b). However, more critical for their adaptation to cold environments than T_{opt} is their competitiveness at low *in situ* temperatures. The comparison of growth rates at 0°C to those at T_{opt} yielded for all strains high relative rates of 24 to 41% (Table 1). For comparison, the only other two sulfate reducers known to grow at 0°C, *Desulfobacter hydrogenophilus* (Widdel, 1987) and "*Desulforhopalus vacuolatus*" (Isaksen and Jørgensen, 1996), had relative growth rates at 0°C of only 2 and 8% of those at T_{opt}, respectively. Furthermore, growth rates of strains LSv514 and LSv21 at 0°C were 3-5 fold higher than those of "*Desulforhopalus vacuolatus*" (Isaksen and Jørgensen, 1996), although T_{opt} of these strains were the same.

The significance of T_{opt} for the competitiveness of bacteria at low environmental temperatures is a topic of dispute. Harder and Veldkamp (1971) showed in chemostat experiments with an obligately psychrophilic

Pseudomonas sp. and a facultatively (moderately) psychrophilic *Spirillum* sp. that the obligate psychrophile outgrew the facultative psychrophile at -2°C and that the opposite was true at 16°C. The authors concluded that obligate psychrophiles have higher growth rates at low temperatures than facultatively (moderately) psychrophiles and are hence superior in cold environments. However, a correlation between T_{opt} and competitiveness at low temperatures could not be found in chemostat experiments with two facultatively psychrophilic bacteria having different temperature optima (20 and 24°C respectively [Nedwell and Rutter, 1994]), since the "less psychrophilic" strain (i.e. the one with the higher T_{opt}) would overgrow the "more psychrophilic" strain at temperatures below 12°C. Obligately psychrophilic bacteria appear to be dominant in certain sea ice microbial communities (Delille, 1992; Helmke and Weyland, 1995; Bowman *et al.*, 1997a), but the reason for this dominance remained unclear because growth rates of obligate psychrophiles at very low temperatures (-5 to 3°C) were not different from those of facultative psychrophiles (Helmke and Weyland, 1995). Similar results were found in our experiments: growth rates of strains LSv21 and LSv54 below 10°C were comparable, whereas T_{opt} was about 8°C different (Fig. 2 and 4), thus suggesting that there is no clear correlation between T_{opt} and growth rates at low temperatures.

Strains LSv21, LSv54 and LSv514 were all grown on lactate as carbon and energy source and had doubling times (t_d) at T_{opt} between 19 and 33 h. Growth was, therefore, only slightly slower than in mesophilic marine sulfate reducers (t_d=11-25 h) or in several *Desulfovibrio* species (t_d=19-22 h) grown at 28°C (Brysch *et al.*, 1987; Lien *et al.*, 1998; Sass *et al.*, 1998). In contrast, strains ASv26, grown on acetate, and PSv29, grown on propionate, had much lower growth rates than isolates on lactate. At 10°C the doubling time of ASv26 was about 170 h or one week. However, among mesophilic SRB, only *Desulfobacter* spp. grow rapidly on acetate with doubling times of about 20 h

(Widdel and Pfennig, 1981). Phylogenetically, ASv26 is only distantly related to *Desulfobacter* spp. and its closest mesophilic relative, *Desulfosarcina variabilis* (Knoblauch *et al.*, 1999b) also grows slowly on acetate (Widdel, 1980). PSv29 grew with a doubling time of 140 h at 7°C, which is much slower than the mesophilic propionate oxidizing *Desulfobulbus* strains (10-12 h at 30-35°C, [Widdel and Pfennig, 1982; Samain *et al.*, 1984; Lien *et al.*, 1998]). Furthermore, growth on carbon sources other than propionate was not faster (data not shown), indicating that high doubling times were not due to unsuitable carbon sources. Widdel *et al.* (1983) observed doubling times of 100 h at 28-30°C in *Desulfonema* strains when they were grown with acetate on the same medium we used, but growth rates could be raised more than 3 fold by the addition of anaerobic fermented yeast extract and manure. Hence, one reason for slow growth of ASv26 and PSv29 could be the lack of complex growth stimulating substances possibly present in the sediment but not in our defined medium.

Temperature response of sulfate reduction rates. Sulfate reduction rates of all psychrophilic isolates increased exponentially from below T_{min} (-1.8°C) to above the optimum temperature for growth. The data were fitted using the Arrhenius equation and the apparent activation energies (E_a) were calculated for each strain. E_a values are a measure of the rate increase of a given process (e.g. sulfate reduction) as a function of temperature. The higher the E_a values are, the steeper is the increase of process rates. E_a values of the different strains were nearly the same (54-59 kJ/mol) even though the temperature range used for the calculations differed considerably (Table 1). Sagemann *et al.* (1998) measured the temperature response of the sulfate-reducing community in the same sediments from which the studied strains were isolated. They determined E_a values between 46 and 74 kJ/mol (mean 57 kJ/mol) in a temperature range of -2 to 25°C. These values are similar to those found for our pure cultures.

Coincident apparent activation energies mean that sulfate reduction responds similarly to increasing temperatures in the sediment and the pure cultures and hence supports the interpretation that the studied isolates are members of the SRB community active at *in situ* conditions.

Literature data on E_a of SRB in pure cultures are scarce. From specific sulfate reduction rates between 0 and 30°C of a *Desulfovibrio desulfuricans* strain (Kaplan and Rittenberg, 1964), we calculated E_a values of 74 and 85 kJ/mol; a similar value of 86 kJ/mol was reported by Isaksen and Jørgensen (1996) for "*Desulforhopalus vacuolatus*", a moderately psychrophilic SRB isolated from a temperate estuary (Isaksen and Teske, 1996). E_a values of the psychrophiles from polar sediments are lower, indicating that their energy metabolism is less affected by decreasing temperatures than that of their counterparts from temperate environments.

Highest sulfate reduction rates (SRR) occurred in all strains at temperatures above T_{opt} (Fig. 1-3 and 4), but whereas LSv54 and LSv514 were still able to grow at these temperature (2-5°C above T_{opt}), LSv21 and ASv26 were not (7-9°C above T_{opt}). A difference between the temperature of highest metabolic rate and highest growth rate was reported previously for aerobic psychrophilic bacteria (Christian and Wiebe, 1974; Harder and Veldkamp, 1968) as well as for "*Desulforhopalus vacuolatus*" (Isaksen and Jørgensen, 1996). The significance of this difference in bacteria living at permanently low temperatures is not clear, but in contrast to growth, the temperature response of respiration depends on a more restricted part of the enzymatic machinery in the cell. Pure enzymes from psychrophilic bacteria were shown to have highest catalytic rates at 15-25°C above T_{opt} of the whole organism (Hamamoto *et al.*, 1994; Feller *et al.*, 1995). Such high temperature optima of enzymes in psychrophilic bacteria might explain the discrepancy between T_{opt} for growth and respiration.

Temperature response of growth yield. Although strains ASv26 and PSv29 grew very slowly on the substrates on which they were isolated (acetate and propionate, respectively), growth yields of ASv26 (3.5-4.9 g per mol acetate) were similar to those reported for the much faster growing, acetate-oxidizing, mesophilic *Desulfobacter* strains (4.5-4.8 g per mol acetate [Widdel and Pfennig, 1981; Isaksen and Jørgensen 1996; Lien and Beeder, 1997]). The same was the case for growth yields of PSv29 (3.2 g per mol propionate) relative to those of mesophilic propionate oxidizing *Desulfobulbus* strains (4.3 to 5.5 g per mol propionat [Widdel and Pfennig, 1982; Lien *et al.*, 1998]). LSv54 had an almost constant growth yield between T_{min} and T_{opt} (mean of 4.2 g per mol lactate) but in LSv514 and LSv21 yield increased with decreasing temperatures and was highest (5.6 or 6.5 g per mol lactate, respectively) at the lowest growth temperatures (Fig. 2c and 5c). Literature data on growth yields of mesophilic SRB with lactate as carbon source differ strongly. Lowest values of about 2 g per mol have been reported for *Desulfovibrio africanus* (Traore *et al.*, 1982) and *Desulfovibrio gigas* (Magee *et al.*, 1978). Most literature data are in the range of 4-8 g per mol (Magee *et al.*, 1978; Traore *et al.*, 1981; Traore *et al.*, 1982; Pankhania *et al.*, 1986; Kremer *et al.*, 1988; Sass *et al.*, 1998), and highest yields of 10 g per mol were reported for *Desulfovibrio desulfuricans* strain Canet 41 (Senez, 1962). The growth yields of all investigated psychrophilic SRB measured at T_{min} are hence in the middle range of previously reported values for mesophilic SRB at T_{opt}.

Growth yield of a culture might be affected by the growth rate, particularly when it is very low (Gottschal, 1986). Since in batch incubations growth rates cannot be kept constant at different temperatures, this additional variable must be considered. However, decreasing growth rates tend to cause lower growth yields, because the relative energy requirements for maintenance increase when growth rates slow down (Stouthamer and Bettenhaussen, 1973).

Consequently, lower growth rates in batch incubations at lower temperatures can hardly be responsible for the observed increase in growth yield.

The few earlier studies on temperature response of growth yields in mesophilic SRB indicate that these are highest close to T_{opt} and decrease strongly at T_{min} (Isaksen and Jørgensen, 1996; Sass *et al.*, 1998). In contrast, the growth yield in psychrophilic SRB tends to increase when temperature decreases (LSv514, LSv21) or at least to stay nearly constant between T_{opt} and T_{min} (Isaksen and Jørgensen, 1996; this study). Similar results were also reported for other psychrophilic bacteria. Herbert and Bell (1977) found increasing growth yields with decreasing temperatures in a psychrophilic *Vibrio* sp., whereas the yield of a psychrophilic *Pseudomonas* sp. stayed almost constant between T_{opt} and T_{min} (Harder and Veldkamp, 1967). Two moderately psychrophilic aerobes responded differently to decreasing temperatures (Nedwell and Rutter, 1994). One strain showed a constant growth yield between T_{opt} and T_{min}, whereas in the other strain growth yield was more strongly affected by the initial substrate concentration in the medium than by temperature. A possible explanation for decreasing growth yield with increasing temperature is a less efficient coupling of respiration and energy production in cells of strains LSv21 and LSv514 growing at higher temperatures. The same explanation was given previously to explain low growth yields in mesophiles (Senez, 1962; Ng, 1969) as well as in thermophiles (Coultate and Sundaram, 1975). Furthermore, a temperature dependence of maintenance energy was demonstrated by Herbert and Bell (1977) who showed that the maximum growth yield of a *Vibrio* sp. at the lowest incubation temperature was due to minimum maintenance energy requirements at that temperature. A similar temperature effect on maintenance energy, and hence, on growth yield was also reported for a mesophilic *Aerobacter* strain (Topiwala and Sinclair, 1971).

The competitiveness of Arctic psychrophiles in their natural environment must depend on a combined adaptation to permanently low temperature and to

the low nutrient concentrations of marine sediments. In addition to growth rates and substrate affinity, growth yield also affects the outcome of competition when substrates are limiting (Gottschal, 1986). Our results indicate that growth yield is an important but often overlooked factor in temperature adaptation of bacteria. High growth yields at low ambient temperature may be a characteristic of cold-adapted bacteria and may provide the psychrophiles with a competitive advantage at low temperature.

EXPERIMENTAL PROCEDURES

Origin of organisms. All strains were isolated from Arctic marine sediments at Svalbard. Strains LSv21, ASv26, and PSv29 originated from Hornsund sediment (76°58.2' N, 15°34.5' E) with a bottom water temperature of 2.6°C. Strains LSv54 and LSv514 were isolated from Storfjord sediment (77°33.0' N, 19°05.0' E) with a bottom water temperature of -1.7°C. Further information about sampling sites are given in Glud *et al.* (1998). Strains LSv21, LSv54 and LSv514 were isolated on lactate (20 mM), strain ASv26 on acetate (15 mM), and strain PSv29 on propionate (20 mM). The incubation temperature during isolation of all strains was 4°C, except for strain LSv54, which was isolated at 10°C. Strains LSv21 and ASv26 will be described as two species of the new genus *Desulfofrigus*, strain PSv29 as the only member of the new genus *Desulfofaba*, and strains LSv54 and LSv514 as different species of the new genus *Desulfotalea* (Table 1). The complete physiological characterization and the taxonomic affiliation of all strains will be presented elsewhere (Knoblauch *et al.*, 1999b).

Cultivation of bacteria. For cultivation of psychrophilic SRB the sulfide reduced artificial seawater medium described by Widdel and Bak (1992) was modified as described in Knoblauch *et al.* (1999b). Marine salt concentrations allowed optimum growth of all investigated strains except LSv54 which has a

salt optimum of 1 %. That strain was consequently grown on 10 g NaCl per liter medium. Stock cultures and precultures for temperature experiments were incubated at the temperature used for isolation of the respective strains, 4 or 10°C. All strains were grown on the carbon substrate used for isolation.

Growth determination and direct cell counts. Since all isolates, except LSv54, grew in aggregates, cultures had to be homogenized prior to optical density (OD) measurements for growth determinations or direct cell counts. Homogenization was performed by an ultrasonic probe (HD200, Bandelin, Berlin, Germany) at the lowest intensity. Microscopic controls revealed that cells were not damaged by this treatment. Cells were counted under the light microscope in a Neubauer chamber (chamber depth 20 µm). OD was measured in a spectrophotometer (UV-1202, Shimadzu, Duisburg, Germany) at 580 nm. Specific growth rates were calculated in exponentially growing cultures by a linear regression of ln OD_{580} as a function of time.

Growth yield determination. Growth yield is expressed as the cellular biomass produced per amount of carbon substrate dissimilated. For growth yield determination all cultures were sampled at the beginning of the experiments and in the late exponential growth phase. Biomass produced was calculated from the difference of carbon content in the cultures at the beginning and at the end of experiments. Carbon was converted to dry weight by multiplication with a factor of 2.1, assuming that the overall composition of cellular biomass is $C_4H_7O_3$ (Widdel and Pfennig, 1981). The amount of substrate assimilated into biomass was calculated from the amount of biomass produced (as $C_4H_7O_3$) and subtracted from the total amount of carbon substrate utilized to yield the amount of carbon substrate dissimilated.

Sulfate reduction rate measurements. Sulfate reduction rates of bacterial pure cultures were measured with the radiotracer method (Sorokin, 1962; Jørgensen, 1978). Pure cultures were grown in 1 liter of medium to the exponential growth phase. At the beginning of most experiments, cultures had

produced 2-3 mM sulfide. A known culture volume of approx. 10 ml was transferred aseptically to sterile 15 ml Hungate tubes, the gas phase was flushed with CO_2/N_2 (10/90, v/v) to remove oxygen, and the tubes were sealed with butyl rubber stoppers. Cultures were preincubated for 1 h in a temperature gradient block to establish a constant temperature. Afterwards 200 kBq $^{35}SO_4^{2-}$-tracer (Amersham, Braunschweig, Germany) was injected through the rubber stopper and mixed into each culture. After 4 to 6 hours of incubation, sulfate reduction was stopped by injection of 1 ml zinc-acetate (20%) through the stopper and tubes were vigorously shaken to trap all sulfide present in the headspace. The ^{35}S-labelled sulfide formed was distilled with 6 M HCl in a single-step distillation (Fossing and Jørgensen, 1989). A 5 ml sample from the distillation was mixed with 10 ml scintillation liquid (Ultima Gold XR, Canberra Packard, Dreieich, Germany) and counting of $^{35}SO_4^{2-}$ and $Zn^{35}S$ was performed by a liquid scintillation counter (Canberra-Packard 2400TR). Sulfate reduction rate blanks were made by stopping the biological activity in a culture with 1 ml Zinc-acetate prior to addition of the $^{35}SO_4^{2-}$-tracer. Blanks were then treated as described above and the radioactivity of blanks was subtracted from the radioactivity of the samples. Sulfate reduction rates were calculated according to Jørgensen (1978).

Temperature incubations. Temperature response of growth and growth yield was determined for strains ASv26, LSv21, PSv29 and LSv514 in 250 ml cultures incubated in duplicates at 10 different temperatures. Since latter cultures tend to grow in clumps, cultures were placed on a stirrer in temperature controlled incubators and stirred with a magnetic bar, thereby keeping the cultures as homogeneous as possible. At defined time intervals samples for OD and sulfide measurements were withdrawn, and temperature was measured in a bottle of water, placed on the stirrers adjacent to the culture vessels. Since strain LSv54 did not build clumps, it was grown in 15 ml tubes and the OD of cultures was measured directly in a spectrophotometer (UV-1202, Shimadzu). Triplicate

cultures were incubated in an insulated temperature gradient block (Sagemann *et al.*, 1998) between -1.8 and 20°C. At the beginning and end of the experiments additional samples for volatile fatty acid analysis and cellular carbon determinations were taken. The temperature response of sulfate reduction in strains ASv26, LSv54, and LSv514 was determined by incubating triplicate cultures in a temperature gradient block at 30 different temperatures between -3 and 40°C. Experiments with LSv21 were not run in triplicate as described above, because the cells of LSv21 slowly started to lyse during the transfer of the 1 l culture to the individual Hungate tubes (see above). Thus only up to 30 Hungate tubes could be prepared at a time that would produce sulfate reduction rates unaffected by cell lysis (data not shown). A repeat run of this experiment gave the same results. In Fig. 2b the results of one representative experiment are presented.

Chemical determinations. Culture samples for sulfate determination were filtered through a 0.45 µm filter (Acrodisc 4, Gelman sciences, Michigan, USA) and measured by unsuppressed anion chromatography (Waters HPLC system, Waters Association, Milford, USA, consisting of a 510 HPLC pump, a WISP 712 autosampler, an IC-Pak anion exchange column (50 x 4.6 mm), and a 430 conductivity detector). The eluent was a 1 mM isophthalate buffer in 10% (v/v) methanol/water (pH 4.5). Cell carbon was determined with a CHNS analyzer (Cutter and Radford-Knoery, 1991). A defined culture volume (5-20 ml) was filtered on two GF/F-filters (1.28 cm, Frisenette, Ebeltoft, Denmark) placed in two filter holders mounted in series. The second filter was used as a blank. The filters were washed once with a salt solution (20 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$ per liter water) and then dried in a stream of sterile-filtered air. The filters were placed in tin capsules and 50 µl distilled water and 50 µl HCl (50 mM) were added to dissolve the bicarbonate. After two hours the filters were dried over night at 105°C and analyzed with a CHNS analyzer (NA1500N, Fisons, Rodano, Italy). Volatile fatty acids and lactate were determined by ion

exclusion chromatography with a HPLC system (Sykam, Gilching, Germany) and a refractometer (ERC-7515, ERC. INC., Alteglofsheim, Germany) as detector. The components were separated on a Sarasep WA1 column (300 x 7.8 mm) at 60°C with H_2SO_4 (15 mM) as eluent. The flow was adjusted to 0.6 ml min^{-1}. 50 µl of a 0.45 µm filtered sample (Acrodisc 4, Gelman Sciences, Michigan, USA) was injected on the column. Sulfide was measured by the methylene blue method (Cline, 1969).

Calculations and determination of T_{opt}. Temperature characteristics of psychrophilic SRB were described by the Arrhenius function, Rate = $A \cdot \exp(-E_a \cdot [R \cdot T]^{-1})$, where A = a constant, E_a = apparent activation energy or temperature characteristic of the reaction, R = gas constant and T = absolute temperature in K. The function was fitted to sulfate reduction rates measured at different temperatures by non-linear regression using KaleidaGraph 3.0.4 (Abelbeck Software). The Arrhenius function was used, because it provided a good fit of the data and has proven useful to describe temperature response of sulfate reduction (Abdollahi and Nedwell, 1979; Westrich and Berner, 1988; Sagemann *et al.*, 1998; Crill and Martens, 1987; Isaksen and Jørgensen, 1996). To calculate T_{min}, T_{opt} and T_{max} values a nonlinear model described by Ratkowsky *et al.* (1983) was applied to the data. However, since the function did not fit satisfactorily to the measured growth rates, T_{opt} values were estimated from the temperatures at which highest growth rates were determined.

3. SUMMARY

Manuscript (1)

The background of the study presented in the first manuscript, *Community size and metabolic rates of psychrophilic sulfate reducing bacteria in Arctic marine sediments* were findings of similar sulfate reduction rates in permanently cold, polar sediments as well as in temperate environments and the absence of any cultivated sulfate-reducing organism that is able to grow in polar sediments.

Sulfate reduction rates in two permanently cold shelf sediments at Svalbard, Arctic Ocean, were determined and sulfate-reducing bacteria were counted by the cultivation-dependent most probable number (MPN) method. Besides the estimation of the community size of a certain group of bacteria this method allows a subsequent isolation of the most abundant cultivable organisms. Furthermore, sulfate reduction rates per cell (i.e. specific sulfate reduction rates) of 19 different psychrophilic isolates were measured at the *in situ* temperature of the respective sampling sites. The specific sulfate reduction rates of 9 different mesophilic marine sulfate-reducing bacteria were measured at 4-13°C, which are temperatures normally encountered in temperate sediments, and compared to those of psychrophiles.

MPN-counts indicated that the investigated sediments are dominated by sulfate reducers that are not able to grow at 20°C, i.e. by psychrophiles. Furthermore, all isolated strains except of one, grew fastest at temperatures below 20°C. However, more relevant for the adaptation to cold environments is the lowest temperature at which growth occurred which was in all investigated strains the freezing point of sea water, -1.8°C. Specific sulfate reduction rates of psychrophiles at -1.7 and 2.6°C (*in situ* temperatures of the sampling sites) were similar to those of mesophiles at 6-10°C higher temperatures.

The results of this study showed for the first time, that psychrophilic sulfate-reducing bacteria are present in polar sediments and that they are adapted to

permanently low temperatures by considerably higher specific sulfate reduction rates than their mesophilic relatives at similar low temperatures. Comparable sulfate reduction rates at both permanently cold and moderate sediments might be explained by a similar community size and similar specific sulfate reduction rates of psychrophiles at cold and mesophiles at moderate temperatures.

The second manuscripts is closely related to the first, since both describe studies on the sulfate reducing community in the same sediments. In the first study quantification of sulfate reducers was achieved by cultivation-dependent MPN enrichments. Cultivation-dependent methods are still the only methods that allow the isolation of the most abundant cultivable organisms. However, these methods are known to be inherently selective and underestimate the active population of microorganisms up to several orders of magnitude.

Manuscript (2)

In the second manuscript *Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine Arctic sediments* 13 psychrophilic sulfate-reducing bacteria were phylogenetically classified. The abundance of different groups of sulfate-reducing bacteria was determined by cultivation independent 16S rRNA slot-blot hybridizations and a group of psychrophilic sulfate-reducing isolates was quantified with a new 16S rRNA oligonucleotide probe.

The results showed that the new phylogenetic group of psychrophilic isolates made up 23-30% of the total detected sulfate reducer community in the most active sediment layers. Furthermore, high numbers of sulfate-reducing bacteria identical to three of the isolated psychrophiles (strains LSv24, LSv23 and LSv53) were found by denaturing gradient gel electrophoresis (DGGE) in both sampling sites.

The results of this study confirm that the isolated psychrophilic sulfate-reducing bacteria are quantitatively important representatives of the sulfate reducing community.

Manuscript (3)

The third manuscript, *Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of Desulfofrigus oceanense gen. nov., sp. nov., Desulfofrigus fragile sp. nov., Desulfofaba gelida gen. nov., sp. nov., Desulfotalea psychrophila gen. nov., sp. nov. and Desulfotalea arctica sp. nov.*, reports the detailed description of five new psychrophilic sulfate-reducing bacteria. The described strains were isolated on the most common fermentation products (acetate, propionate, lactate) which are incompletely oxidized to acetate by *Desulfofaba gelida*, strain PSv29, *Desulfofrigus fragile*, strain LSv21, *Desulfotalea psychrophila*, strain LSv54, and *Desulfotalea arctica*, strain LSv514 and completely to CO_2 by *Desulfofrigus oceanense*, strain ASv26. Hence the most important substrates of sulfate reduction in temperate sediments are also oxidized by the psychrophilic isolates. All strains except LSv54, have a marine salt optimum and strains ASv26, LSv21, LSv54 and LSv514 can grow by iron(III) reduction. The temperature optima for growth of the different strains is between 7 and 18°C. Chemotaxonomic variables, such as cellular fatty acid pattern, menaquinone content and utilized carbon substrates, and phylogeny based on the16S rRNA sequence showed good agreement and the new isolates were assigned to three new genera of the δ *Proteobacteria*. The results of this study demonstrate, that the psychrophilic sulfate-reducing bacteria in permanently cold sediments differ phylogenetically from their counterparts in moderate environments but suggest that they occupy the same ecological niche as mesophiles in moderate environments.

Manuscript (4)

The fourth manuscript, *Effect of temperature on sulfate reduction, growth rate, and growth yield in five psychrophilic sulfate-reducing bacteria from Arctic sediments,* is a detailed description of the temperature adaptation of the five isolates described in the third manuscript. All investigated strains have temperature optima below 20°C and *Desulfofaba gelida*, strain PSv29 has the lowest growth optimum, 7°C, reported for any anaerobic prokaryote. Highest metabolical activity were found in all strains at temperatures above the optimum temperature of growth and *Desulfofrigus oceanense*, strain ASv26, and *Desulfofrigus fragile*, strain LSv21, cannot grow at the temperatures where sulfate reduction rates are highest. Growth rates at 0°C, which is close to the *in situ* temperature of their natural environment (-1.7 and 2.6°C respectively) were still 25-4% of those measured at the optimum temperature for growth. Whereas growth and respiration rates decreased with decreasing temperatures growth yield increased in *Desulfofrigus fragile*, strain LSv21, and *Desulfotalea arctica*, strain LSv514, and was highest at the lowest temperature. Growth yield of *Desulfofrigus oceanense*, strain ASv26, *Desulfofaba gelida*, strain PSv29, and *Desulfotalea psychrophila*, strain LSv54, was almost constant between the optimum temperature of growth (7-18°C) and the minimum growth temperature (-1.8°C). This temperature response of growth yield is different from that in mesophilic sulfate-reducing bacteria where growth yield was found to be lowest at the minimum temperature of growth. The results of this study demonstrate that the isolated psychrophilic sulfate-reducing bacteria are adapted to permanently cold environments by relatively high potential growth rates at the low *in situ* temperatures. Highest growth yields at the lowest temperatures appear to be a further adaptation to cold environments. Besides growth rates and substrate affinity, growth yield affects the outcome of competition among different microorganisms when carbon substrates are limiting, which is the case in most marine sediments. Highest growth yield at low

in situ temperatures is hence a competitive advantage for low temperature adapted sulfate reducers living in cold sediments.

OUTLOOK

In the present study it was shown that the sulfate-reducing community in polar sediments comprises organisms that are different from those isolated from temperate sediments. Psychrophilic sulfate reducers living at permanently low temperatures are adapted to their cold environment by having relatively high metabolic activities, growth rates, and growth yields at temperatures below 0 °C. However, the results presented here can only be seen as a first step and further studies on environmentally important sulfate reducers are highly needed. As pointed out previously, pure cultures of sulfate reducers that are active at common environmental temperatures (<15 °C) have long been lacking and, consequently, pure culture experiments were almost exclusively made at temperatures irrelevant for most marine sediments. It remains unclear, to what extent investigations with previously available laboratory strains can be related to the natural environment. Future attempts to isolate sulfate-reducing bacteria should be made at or close to the *in situ* temperature of the sampling sites to obtain organisms that can be active at *in situ* conditions.

Laboratory culture experiments will always be artificial and can never reflect the conditions encountered by microorganisms in their environment. However, if we want to understand what adaptation of microorganisms to an environmental parameter such as temperature means, we must try to resembled nature as close as possible. Temperature is one important variable that affects competition between microorganisms. Another variable is the substrate concentration. Whereas temperature can be controlled easily in laboratory batch experiments, substrate concentration cannot. The laboratory experiments reported in the present study were made in batch cultures that contain about 1000-fold higher

substrate concentration than found in the environment. Growth rates measured in these experiments are therefore only potential rates and much higher than those under natural conditions. On the other hand, the actual growth rate of a microorganism at a certain substrate concentration is one of the critical parameters for the outcome of competition among organisms. The only way to grow organisms continuously at low substrate concentrations is by the use of chemostat cultures. Such chemostat experiments make it possible to study one bacterial strain or the competition among different strains at conditions close to those found in the environment. Even though these experiments are costly and time consuming, especially when made with slow-growing organisms at low temperatures, the results will give valuable insights into the ecological physiology and, thus, the role of the studied organisms in the natural community.

The combination of cultivation dependent and molecular methods was shown to be a successful approach for investigations of new organisms in natural communities. Therefore, studies on microbial ecology should combine the advantages of both methods rather than rely only on one of both.

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