Polar Biol (2001) 24: 252–257
DOI 10.1007/s003000000205

ORIGINAL PAPER

Ralf Petri · Johannes F. Imhoff

Genetic analysis of sea-ice bacterial communities of the Western Baltic Sea using an improved double gradient method

Accepted: 16 October 2000 / Published online: 19 January 2001

Abstract The bacterial diversity of sea ice from Kiel Bight obtained during the rare event of solid ice cover in spring 1996 was analysed by molecular genetic approaches using an improved double gradient denaturing gradient gel electrophoretic method (DG-DGGE) to separate 16S rDNA fragments of approximately 500 bp. The excellent separation of individual bands within these gradient gels allowed us to obtain sequence information and to allocate the phylogenetic position of representative bacteria from the sea ice. The band pattern of the gradient gels revealed a vertical stratification of the bacterial species distribution within the ice and the presence of characteristic bacteria for each layer. According to their 16S rDNA sequences, major bands of the gradient gels represented bacteria closely related to fermenting species of the genera *Propionibacterium* and *Bacteroides* and to anoxygenic phototrophic purple sulfur bacteria (Chromatiaceae). Their abundance in horizons of the inner ice core may indicate the existence of oxygen-deficient and anoxic zones or niches and possible primary production by anoxygenic photosynthesis within the investigated Baltic Sea sea ice. This is the first phylogenetic evidence of the presence, and most probably the development, of phototrophic purple sulfur bacteria in sea ice.

Introduction

A solid ice cover in the western Baltic Sea over a prolonged period is a rare event and occurred recently in late winter 1995/1996. At the end of January 1996 the first ice formation took place, and during the following weeks a solid ice cover developed that lasted for more than 2 months.

Little information is available on bacterial communities of the Baltic Sea sea ice. With regard to total bacterial numbers and biomass, presence of distinct morphological types of bacteria and to bacterial production, certain similarities were found between bacterial communities of sea ice from the Baltic Sea and those found in polar regions (Mock et al. 1997). Nutrient concentrations within sea ice from the Baltic Sea exceeded the values known from Arctic and Antarctic sea-ice samples, but were comparable to values estimated in the Gulf of Bothnia (Norrman and Anderssen 1994). The brine salinity from the Baltic Sea sea ice was low in comparison to polar sea ice (conductivity ratio of 3.5% KCl/sample of 5.2) (Mock et al. 1997).

Although bacterial assemblages within the sea ice and in the underlying water revealed clearly different morphological cell types (Mock et al. 1997), the diversity of these sea-ice communities has not been investigated so far and the question of whether specific bacterial communities develop within the young sea ice of the Baltic Sea has not yet been answered.

Due to the lack of annual sea-ice cover in this region and the fact that bacterial activities and abundance are low in the initial stages of ice formation (Grossmann and Dieckmann 1994), it can be supposed that specific sea-ice bacteria are absent in the Western Baltic Sea, although the presence of cold-adapted and psychrophilic bacteria is not unlikely (Helmke and Weyland 1991; Mock et al. 1997). If such bacteria are present, they may develop into a characteristic ice-adapted bacterial community due to their selective advantage at the low temperatures within the ice.

In the present study, we investigated the bacterial diversity of a depth profile through the well-developed sea-ice cover in the Western Baltic Sea on 15 March 1996, when it was approximately 6 weeks old and 70 cm in thickness (Mock et al. 1997). Molecular genetic approaches and an improved gel electrophoretic

R. Petri · J. F. Imhoff (✉)
Institut für Meereskunde, Marine Mikrobiologie,
Düsternbrooker Weg 20, 24105 Kiel, Germany
E-mail: jimhoff@ifm.uni-kiel.de
Fax: +49-431-565876

separation method were applied to analyse DNA extracted from the natural samples.

Materials and methods

Sea ice and brine samples

The ice cores were taken at the shallow near-shore station "Strander Bucht" in March 1996 using a 3′-Sipre ice auger driven by a power drill as described by Mock et al. (1997). The cores were melted overnight in the same volume of 0.2 μm prefiltered seawater (under-ice water of the sampling site) at 3 °C to avoid osmotic stress.

Extraction of total DNA from ice samples

Total DNA was extracted from the melted sea ice using the QIAamp Kit (Qiagen, Hilden, Germany). The melted ice samples (20 ml each) were filtered through 0.1-μm cellulose acetate filter. The filters were transferred into a 10-ml polypropylen centrifugation tube, overlayed with 180-μl lysis-buffer [20 mg/ml lysozym; 20 mM Tris-HCl (pH 8.0); 2 mM EDTA; 1.2% Triton] and incubated for 30 min at 37 °C with shaking every 5 min. DNA was then extracted with spin columns from the QIAamp Kit according to the manufacturer's manual.

Primer selection

The most useful nucleotides of the 16S ribosomal DNA to distinguish even between closely related species are those at the beginning of the gene including three hypervariable regions. Therefore, we used universal bacterial primers corresponding to the bases 9–27 (5′-Start primer) and 518–534 (3′-534 primer) for the amplification of the desired fragments. In addition, based on 16S rDNA sequences of various *Pseudomonas* species from the EMBL database aligned with the Clustal program package, a primer specific for the genus *Pseudomonas* was designed. The specificity of the primer was confirmed using ARB (Strunk and Ludwig 1999). This primer is located in the region of 624–642 according to the *Escherichia coli* enumeration and has the sequence: 5′-TTT TGG ATG CAG TTC CCA-3′ (Psd642B). For the DG-DGGE analysis, we designed the following GC-clamp which was attached to the 5′-Start primer: 5′-GCCCGCCGCGCCCCGCGCCCCGCCCGCCGCCCCCGC-CCG.

The primer used for specific amplification of the full-length 16S rDNA from environmental DNA corresponding to band 13 was designed on the basis of the sequence obtained from this DGGE band: 5′-CTG CTA TAG GAT GAG CCC A-3′ (position 217 according to *E. coli* enumeration).

PCR amplification

The PCR amplifications were performed as a touchdown-PCR in a total volume of 20 μl containing 2 μl 10 × PCR-Buffer, 125 μm each of desoxyribonucleoside triphosphate, 0.5 U Taq-Polymerase (5.0 U/μl, Stratagene), 10 pmol of each primer (10 pmol/μl) and DNA (10–100 ng). After a denaturation step of 2 min at 94 °C, the annealing temperature was decreased from 65 °C to 50 °C within 15 cycles (Techne-Cycler). The cycles consisted of 30 s elongation at 72 °C, the 40-s annealing step and the 30-s denaturing step at 94 °C. After the annealing temperature reached 50 °C, an additional 20 cycles were performed under identical conditions. For reasons of better comparison, the *Pseudomonas*-specific amplifications were done in two steps. During the first amplification we obtained group-specific products from environmental DNA with the primer combination 5′-Start and 3′-Psd642, which were then used as templates in a second amplification with the DG-DGGE primer combination 5′-Start + GC clamp – 3′-534. The amplification results were analysed on 1% (wt/vol) agarose gels (Biozym), followed by 10 min of ethidium bromide staining (0.5 mg/l).

Double gradient – Denaturing Gradient Gel Electrophoresis (DG-DGGE)

Gradient gel electrophoresis is an established method in the analysis of bacterial diversity in natural environments (Kowalchuk et al. 1997; Muyzer 1999). Shortcomings of this method are insufficient band separation and difficulties in obtaining fragments sufficiently pure to be used in sequence analysis. In order to improve the quality of band separation of amplified DNA fragments in such gels, a powerful double gradient modification (DG-DGGE) of this method described for clinical applications (Cremonesi et al. 1997) was optimised and applied to environmental DNA. It enabled sequence analysis of bands extracted from the gels. The gradient gel electrophoresis was performed with a CBS Scientific DGGE4000-system. DG-DGGE gels were poured as double gradient gels, consisting of an acrylamide gradient from 6 to 8% acrylamide 37.5:1 (v/v) superimposed with a denaturant gradient from 40 to 64% of denaturant [100% denaturant is defined as 7 M urea and 40% formamide (v/v)]. Twenty microlitres of the PCR samples were mixed with 6 μl of the dye solution [0.1% bromphenol blue (w/v), 70% glycerol (v/v)] and the entire sample was applied to the gel. The electrophoresis was performed using 0.5 × TAE (20 mM Tris-HCl pH 7.8, 20 mM Na-acetate, 2 mM Na-EDTA) as buffer at a constant voltage of 100 V (40 mA) and 60 °C for 14 h. Thereafter the gels were stained with ethidium bromide (0.5 mg/l) for 30 min, rinsed with MilliQ water for 30 min, and documented using UV transillumination (312 nm) with a Biometra Biodoc I system.

Extraction of DG-DGGE bands

The excision of the DG-DGGE bands for sequencing was performed in a one-step centrifugation. The acrylamide gel material with the DG-DGGE band was transferred into a 0.6-ml Eppendorf cup prepared with a hole in the bottom and filled with siliconised glass wool. This cup was placed into a 1.5-ml Eppendorf cup and centrifuged at ×10,000 *g* for 5 min at room temperature. One microlitre of the obtained 2–3 μl was used to reamplify the DGGE band.

Reamplification of DG-DGGE bands

To avoid the amplification of possible contaminations during the reamplification step, we designed a primer specific for the 3′-end of the GC-clamp. The amplification was performed with this reamplification primer (GC-Reamp 5′-CGCCGCCCCCGCCCG-3′) and the 3′-534 primer as a touchdown-PCR with the following program: 2 min at 94 °C for initial denaturing, ten cycles of a 40-s annealing step decreasing the temperature from 70 °C to 58 °C (−1 °C per cycle), a 30-s elongation step at 72 °C and a 30-s denaturing step at 94 °C. After the annealing temperature reached 58 °C, an additional 15 cycles were performed under identical conditions. The amplification products were analysed on 1% (w/v) agarose gels (Biozym) followed by 10 min ethidium bromide staining (0.5 mg/l).

Sequencing of the DG-DGGE bands

Sequences of the DGGE bands were obtained from QIAquick-purified (Qiagen, Hilden, Germany) products by cycle sequencing with the SequiTherm sequencing kit (Biozym) and the chain termination reaction (Sanger et al. 1977) using an automated laser fluorescence sequencer (Amersham-Pharmacia). The cycle sequencing reaction consisted of 25 cycles with an annealing temperature of 50 °C, under similar conditions as used in the reamplification step. We used the 5′-Start and the 3′-534 primers, as well as the primers corresponding to the *E. coli* position 337–355 in 5′- and 3′-direction as sequencing primers.

Phylogenetic analysis

Sequences were first compared to the EMBL database using the FastA search program (Pearson and Lipman 1988; Pearson 1990) in order to find related 16S sequences from the database. Nucleotide sequences from DGGE bands and closest relatives from the database were aligned using ClustalW with subsequent manual correction. Missing data and gaps in more than one sequence were treated as missing information. Phylogenetic distances were calculated from the data set according to the algorithm of Jukes and Cantor (1969) by using Dnadist from the Phylip program package (Felsenstein 1989). Phylogenetic trees were inferred from the distance data with global rearrangements from Fitch. Bootstrap analysis of nucleotide sequences was performed with the Paup program with 500 bootstrap resamplings (Swofford and Olsen 1990; Hillis et al. 1996).

Results

Molecular genetic methods were applied to the analysis of bacterial diversity in sea ice from the Western Baltic Sea. An ice core taken in March 1996 was cut into slices; the slices were melted, and DNA was extracted and amplified with primers specific for 16S rDNA sequences of eubacteria and the genus *Pseudomonas* as described in Materials and methods. The obtained amplification products were separated with an improved DG-DGGE method and yielded the band pattern shown in Figs. 1 and 2.

The depth profile through the ice core and underlying water obtained with eubacterial primers (Fig. 1) revealed characteristic band patterns for each horizon and significant differences to the underlying water. Except for two bands related to *Staphylococcus* species (nos. 10, 16) most of the bands present in the water were not detected within the ice core. Only a single band (no. 16) was present as a major component in all horizons. Sequences of characteristic bands (nos. 15, 18) of the 30- to 40-cm horizon were closely related (93–98.5% similarity) to those of *Propionibacterium* species, while the sequence of a major band of the 40- to 50-cm horizon was identical to that of a *Bacteroides* strain (Table 1). The sequences of two major bands of the 30- to 40-cm (no. 12) and the 40- to 50-cm horizon (no. 20), respectively, were related to bacteria from iron-rich habitats. The former was related to *Leptothrix mobilis* (Fig. 3). The latter was identical to a partial database sequence retrieved from an acid mine drainage site of Iron Mountain, California (EMBL: AF047636) and was related ($<94\%$) to *Porphyromonas catoniae* (Willems and Collins 1995; Fig. 3). Sequences of the dominant and two additional bands in the 50- to 60-cm horizon (nos. 13, 21, 22) were related to a group of phototrophic purple sulfur bacteria (Fig. 3, Table 1). Using a specific primer, the 16S rDNA sequence of the bacterium that corresponds to the major band was amplified out of the natural sample in sufficient purity to allow sequence analysis and to obtain an almost complete sequence of the 16S rDNA of this bacterium. The corresponding part of this sequence was identical to that retrieved from the DG-DGGE gel. The 16S rDNA sequence demonstrated a close relationship to *Rhabdochromatium marinum* (94.4% sequence similarity, Table 1).

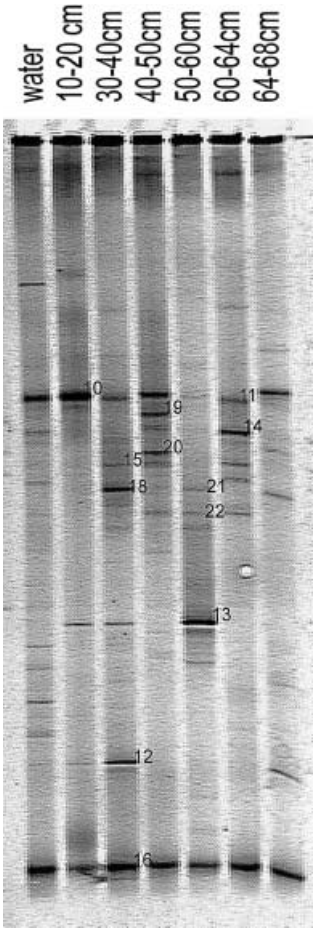

Fig. 1 Depth profile of horizons from Baltic Sea sea-ice core and underlying water obtained with double gradient DGGE of eubacterial amplified 16S rDNA-fragments. The horizons were named according to their distance from the top of the ice core. Bands extracted and sequenced are indicated with a *number* on the *right side* referring to Table 1

Using eubacterial primers, 16S rDNA of *Pseudomonas* species was not amplified to a significant amount and, with the exception of band 14, which is dominant in the 60- to 64-cm layer, none of the major bands was represented by DNA from a *Pseudomonas* species. Therefore, the diversity of this group was analysed by genus-specific amplification. The band pattern obtained from this *Pseudomonas*-specific analysis (Fig. 2) revealed higher diversity in the water than in the ice and demonstrated the presence of dominant bands from the water samples (nos. 4, 5, 7) also within several of the ice horizons. It substantiates the exceptional nature of the 50- to 60-cm layer. Two bands representing bacteria closely related to *Pseudomonas stutzeri* (no. 4) and *Pseudomonas fragi* (no. 7), which were present in all other layers, were absent from this horizon. Instead, several other bands were unique to this horizon. The major bands (nos. 5, 6) were related to a cluster of *Pseudomonas* strains, which included sequences obtained

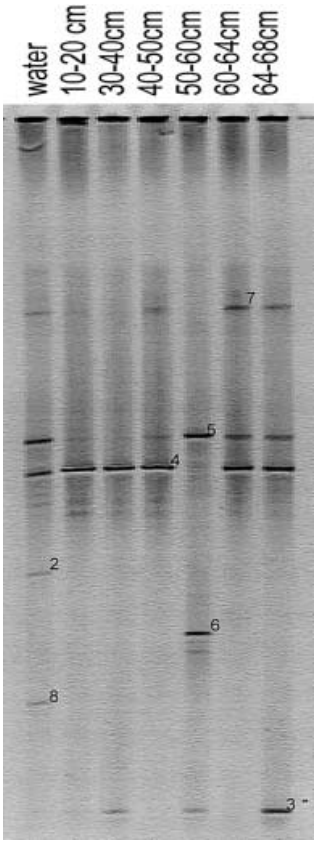

Fig. 2 Depth profile of horizons from Baltic Sea sea-ice core and underlying water obtained with double gradient DGGE of *Pseudomonas*-specific amplified 16S rDNA-fragments. The horizons were named according to their distance from the top of the ice core. Bands extracted and sequenced are indicated with a *number* on the *right side* referring to Table 1

from Japan Trench sediments of more than 6,000 m water depth (sequence similarities of more than 99%; Table 1, Fig. 3).

Discussion

Similar to sea ice from the Antarctic coastal area (Delille 1993), that from the Baltic Sea represents a habitat quite different from the underlying water and enables the development of bacteria different to those characteristic of the water sphere. Only a few bands from the underlying water sample were found also within the ice (Fig. 1). In addition, the present study revealed specific differences of the bacterial communities in different horizons of the sea ice. Although quantitative conclusions from DGGE band patterns cannot be drawn because of several limitations of quantitative DNA extraction and PCR amplification (Muyzer 1999), a major band within a DGGE pattern is highly suggestive of an abundant species in comparison to weak bands. For a particular species and DNA, these limitations are the same in different samples, and therefore the comparative analysis of a set of related environmental samples allows conclusions to be made on the relative abundance of a particular bacterium among these samples. With this in mind, the diversity patterns obtained with eubacterial primers suggest the dominance of different bacterial species within the different sea-ice horizons, as represented by the strong bands in the DGGE lanes.

In particular, in the inner part of the ice core (30- to 64-cm horizons), a few predominant bands were characteristic of each horizon, some of which represented

Table 1 Results of FastA database comparison of sequences from DG-DGGE bands

DG-DGGE band no.	Ice horizon from where DG-DGGE-band was extracted	Next neighbour according to 16S-sequence	Percentage of sequence similarity (%)	Length of sequence (bp)
Eubacterial primer				
21	50–60 cm	*Rhabdochromatium marinum* 5261^T	92.2	501
22	50–60 cm	*Thiorhodovibrio winogradskyi* 6702^T	92.4	499
13	50–60 cm	*Rhabdochromatium marinum* 5261^T	94.4	1348[a]
18	30–40 cm	*Propionibacterium acnes* strain 63597	98.5	471
15	30–40 cm	*P. acnes* strain 63597	93.3	500
16	30–40 cm	*Staphylococcus warneri* ATCC 27836	98.9	466
14	60–64 cm	*Pseudomonas* sp. clone NB1-g	99.2	501
10	10–20 cm	*Staphylococcus epidermidis* ATCC 14990^T	98.8	505
20	40–50 cm	Uncultured eubacterium clone TRA1–16	100	499[b]
11	60–64 cm	*Azospirillum amazonense* DSM 2787	86.2	443
12	30–40 cm	*Leptothrix mobilis* strain Feox-1	92.5	514
19	40–50 cm	*Bacteroides* sp. B-17BO	100	500
Pseudomonas-specific primer				
7	60–64 cm	*Pseudomonas fragi* IAM 12402	99.8	501
2	Water	*Pseudomonas stutzeri* ATCC 17685	96.7	501
3	64–68 cm	*Pseudomonas putida* IAM 1236	95.8	501
8	Water	*Pseudomonas* sp. clone NB1-g	97.5	449
4	40–50 cm	*Pseudomonas stutzeri* ATCC 17598	99.4	501
5	50–60 cm	*Pseudomonas* sp. clone NB1-g	99.2	501
6	50–60 cm	*Pseudomonas* sp. clone NB0.1-H	99.4	518

[a] The original DG-DGGE band was 500 bp in length
[b] The related database sequence had a length of 407 bp

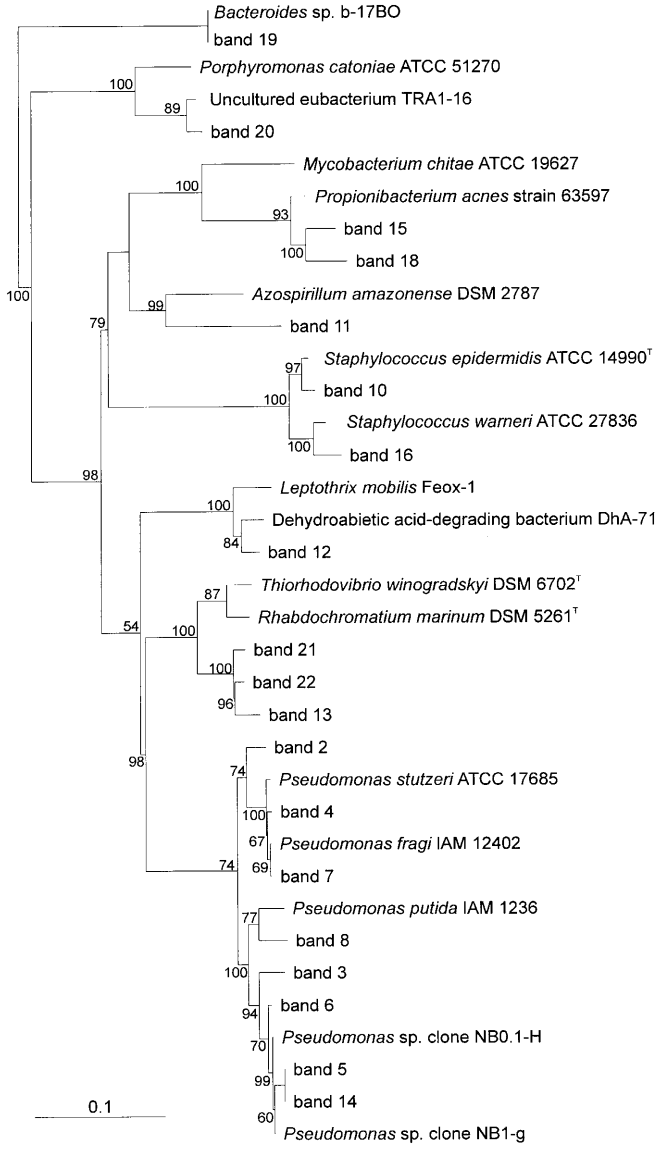

Fig. 3 Phylogenetic tree based on partial 16S rDNA sequence analysis of bands extracted from DG-DGGE and reference sequences obtained from the EMBL database. Bands were numbered according to Figs. 1 and 2. Bands with *numbers* below 10 were obtained from *Pseudomonas*-specific amplification; other bands were obtained from eubacterial amplification. Sequences of 500 bp from the beginning of the 16S rDNA corresponding to the *Escherichia coli* positions 9–534 were aligned using ClustalW. Sequence analysis was performed with the Phylip program package; bootstrap analysis was calculated using PAUP. Distances between two organisms were obtained by summing the lengths of the horizontal branches connecting them, using the *scale* below the tree

bacteria related to species of the genera *Propionibacterium*, *Bacteroides* and to anoxygenic phototrophic purple sulfur bacteria (Table 1, Fig. 3). The close phylogenetic relationship suggests that fermenting and anoxygenic phototrophic bacteria could be among the major representatives of these ice layers, that suboxic or anoxic zones or niches were present and that primary production by anoxygenic photosynthesis may be possible.

A bacterial community was established in the 50- to 60-cm ice horizon that was significantly different from all other horizons and also the underlying water (Figs. 1, 2). Most surprising was the presence of phototrophic purple sulfur bacteria, which quite characteristically develop under anoxic conditions and in the presence of hydrogen sulphide and light. Sequences from three bands of this ice horizon were closely related to a branch of the Chromatiaceae that represents bacteria which are specifically adapted to marine and halophilic habitats (Imhoff et al. 1998). One of these sequences represents the dominant band of this layer obtained in the gradient gels (Fig. 1). This is the first phylogenetic evidence of the presence and, due to the natural abundance, most probably the development of phototrophic purple sulfur bacteria in sea ice. Because the Chromatiaceae form a large phylogenetic cluster of bacteria that is currently without strictly chemotrophic relatives (Imhoff et al. 1998), and the sequences obtained (bands 13, 21, 22) fit very well within this cluster, it is quite likely that the detected sequences belong to a purple sulfur bacterium that finds conditions suitable for growth within the ice cover of the Baltic Sea. Comparison with the whole database of available 16S rDNA sequences from purple sulfur bacteria demonstrated their specific relation to *R. marinum* and *Thiorhodovibrio winogradskyi*, sharing sequence similarities of 94.4% within approximately 1400-bp fragments sequenced after specific amplification. *R. marinum* is an obligately phototrophic and strictly anaerobic bacterium that does not grow chemotrophically (Dilling et al. 1995). This bacterium was found and first described from laminated microbial mats from the Great Sippewissett Salt Marsh at the eastern North American coast, but its occurrence in European marine environments and in particular its presence in the Baltic Sea have so far not been documented. The other purple sulfur bacterium closely related to *R. marinum* and to the sequences retrieved from the sea ice is *T. winogradskyi* (Overmann et al. 1992). This species was originally isolated from a saline lake (Mahoney Lake, Canada) and related strains were found in habitats from the North Sea (Overmann et al. 1992) and the Baltic Sea (J.F. Imhoff, unpublished work). In contrast to *R. marinum*, *T. winogradskyi* is able to grow under microoxic conditions and exhibits high specific respiration rates (Overmann et al. 1992). Both *R. marinum* and *T. winogradskyi* represent typical marine bacteria with optimal development at 1.5–5% and 2–3% NaCl, respectively and form a distinct cluster within the halophilic Chromatiaceae (Imhoff et al. 1998).

We would expect that purple sulfur bacteria that develop within the sea ice have the capacity for both respiratory metabolism and photoheterotrophic growth. These properties would enable them not only to tolerate but also to take advantage of minor quantities of oxygen in the dark, to reduce the oxygen tension further due to their respiratory activities, and to use low molecular weight organic substrates present in the ice as phototrophic electron donors and carbon sources. Such

properties could well be present in a bacterium related to the two mentioned species of marine purple sulfur bacteria.

An additional indication for reduced oxygen concentrations or even anoxic conditions in the central part of the ice is the abundance of bacteria closely related to the fermenting bacteria *Propionibacterium* and *Bacteroides* in the 30- to 50-cm layers (bands 18, 15, 19). An exhaustive database search revealed that the sequence related to *Bacteroides* (band 19) is identical to a *Bacteroides* isolate which was associated with the white truffle, *Tuber borchii* (EMBL: AF070444), but more distantly related (similarity below 90%) to other bacteria from this phylogenetic group (data not shown).

The presence of two major bands (nos. 12, 20) that reveal sequences related to bacteria from iron-rich habitats and are possibly involved in iron transformation is also interesting. At this stage of our knowledge, these sequences provide a clear hint to consider iron transformations as possibly important functions of bacterial sea-ice communities.

The existence of psychrophilic and psychrotrophic bacteria is known from various cold habitats, including sea ice and the deep sea (Rüger 1988; Rüger and Tan 1992), but also temperate environments (Helmke and Weyland 1991). Although it is tempting to assume that bacteria adapted to the sea-ice habitat (and the deep sea) have psychrophilic properties, our sequence data do not allow any conclusion in regard to the cold adaptation of the *Pseudomonas* strains whose sequences have been amplified from the sea ice, even if some of these sequences (band nos. 3, 5, 6, 14) were closely related to clone sequences (NBO.1-H and NB1-g) obtained from extremely deep waters off the coast of Japan with similarities of 99.2 and 99.4% (Yanagibayashi et al. 1999). Proof of this adaptation requires cultivation and growth experiments with the isolated bacteria.

Acknowledgements We thank H.C. Giesenhagen for the disposal of sea-ice samples she obtained in winter 1995/1996, together with T. Mock and K. Meiners.

References

Cremonesi L, Firpo S, Ferrari M, Righetti PG, Gelfi C (1997) Double-gradient DGGE for optimized detection of DNA point mutations. Biotechniques 22: 326–330

Delille D (1993) Seasonal changes in the abundance and composition of marine heterotrophic communities in an Antarctic coastal area. Polar Biol 13: 463–470

Dilling W, Liesack W, Pfennig N (1995) *Rhabdochromatium marinum* gen. nom. Rev., sp. nov., a purple sulfur bacterium from a salt marsh microbial mat. Arch Microbiol 164: 125–131

Felsenstein J (1989) Phylip – phylogenetic interference package (version 3.2). Cladistics 5: 164–166

Grossmann S, Dieckmann GS (1994) Bacterial standing stock, activity, and carbon production during formation and growth of sea ice in the Weddell Sea, Antarctica. Appl Environ Microbiol 60: 2746–2753

Helmke E, Weyland H (1991) Effect of temperature on extracellular enzymes occurring in permanently cold marine environments. In: Rheinheimer G, Gocke K, Hoppe HG, Lochte K, Meyer-Reil (eds) Distribution and activity of microorganisms in the Sea. Kiel Meeresforsch Sonderh 8: 198–204

Hillis DM, Moritz C, Mable BK (1996) Molecular systematics, 2nd edn. Sinauer, Sunderland, Mass

Imhoff JF, Süling J, Petri R (1998) Phylogenetic relationships among the Chromatiaceae, their taxonomic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. Int J Syst Bacteriol 4: 1129–1143

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HM (ed) Mammalian protein metabolism. Academic Press, New York, pp 21–132

Kowalchuk GA, Stephen JR, De Boer W, Prosser JI, Embley TM, Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol 63: 1489–1497

Mock T, Meiners KM, Giesenhagen HC (1997) Bacteria in sea ice and underlying brackish water at 54°26′50″N (Baltic Sea, Kiel Bight). Mar Ecol Prog Ser 158: 23–40

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2: 317–322

Norrman B, Anderssen A (1994) Development of ice biota in a temperate sea area (Gulf of Bothnia). Polar Biol 14: 531–537

Overmann J, Fischer U, Pfennig N (1992) A new purple sulfur bacterium from saline littoral sediments, *Thiorhodovibrio winogradskyi* gen. nov. and sp. nov. Arch Microbiol 157: 329–335

Pearson WR (1990) Rapid and sensitive sequence comparison with FAST and FASTA. Methods Enzymol 183: 63–98

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence analysis. Proc Natl Acad Sci USA 85: 2444–2448

Rüger H-J (1988) Substrate-dependent cold adaptations in some deep-sea sediment bacteria. Syst Appl Microbiol 11: 90–93

Rüger H-J, Tan TL (1992) Community structures of cold and low-nutrient adapted heterotrophic sediment bacteria from the deep eastern tropical Atlantic. Mar Ecol Prog Ser 84: 83–93

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Strunk O, Ludwig W (1999) ARB: a software environment for sequence data. Technische Universität München, Munich http://www.biol.chemie.tu-muenchen.de/pub/ARB/

Swofford DL, Olsen GJ (1990) Phylogeny reconstruction. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer, Sunderland, Mass, pp 411–501

Willems A, Collins MD (1995) Reclassification of *Oribaculum catoniae* (Moore and Moore 1994) as *Porphyromonas catoniae* comb. nov. and emendation of the genus *Porphyromonas*. Int J Syst Bacteriol 45: 578–581

Yanagibayashi M, Nogi Y, Li L, Kato C (1999) Changes in the microbial community in Japan Trench sediment from a depth of 6292 m during cultivation without decompression. FEMS Microbiol Lett 170: 271–279