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Impact of Different In Vitro Electron Donor/Acceptor Conditions on Potential Chemolithoautotrophic Communities from Marine Pelagic Redoxclines

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Anaerobic or microaerophilic chemolithoautotrophic bacteria have been considered to be responsible for CO_2 dark fixation in different pelagic redoxclines worldwide, but their involvement in redox processes is still not fully resolved. We investigated the impact of 17 different electron donor/acceptor combinations in water of pelagic redoxclines from the central Baltic Sea on the stimulation of bacterial CO_2 dark fixation as well as on the development of chemolithoautotrophic populations. In situ, the highest CO_2 dark fixation rates, ranging from 0.7 to 1.4 μmol liter^{-1} day^{-1}, were measured directly below the redoxcline. In enrichment experiments, chemolithoautotrophic CO_2 dark fixation was maximally stimulated by the addition of thiosulfate, reaching values of up to 9.7 μmol liter^{-1} CO_2 day^{-1}. Chemolithoautotrophic nitrate reduction proved to be an important process, with rates of up to 33.5 μmol liter^{-1} NO_3^- day^{-1}. Reduction of Fe(III) or Mn(IV) was not detected; nevertheless, the presence of these potential electron acceptors influenced the development of stimulated microbial assemblages. Potential chemolithoautotrophic bacteria in the enrichment experiments were displayed on 16S ribosomal complementary DNA single-strand-conformation polymorphism fingerprints and identified by sequencing of excised bands. Sequences were closely related to chemolithoautotrophic *Thiomicrospira psychrophila* and *Maorithyas hadalis* gill symbiont (both *Gammaproteobacteria*) and to an uncultured nitrate-reducing *Helicobacteraceae* bacterium (*Epsilonproteobacteria*). Our data indicate that this *Helicobacteraceae* bacterium could be of general importance or even a key organism for autotrophic nitrate reduction in pelagic redoxclines.

Chemolithoautotrophic bacteria play an important role in biogeochemical cycles of aquatic habitats. Molecular hydrogen and reduced nitrogen (NH_4^+ and NO_2^-), sulfur (e.g., H_2S and $S_2O_3^{2-}$), metals (e.g., Fe^{2+} and Mn^{2+}) and carbon (e.g., CO and CH_4) compounds serve as electron donors for these bacteria (37), whereas oxygen and nitrate mostly serve as electron acceptors. CO_2 dark fixation has been determined in different pelagic redoxclines worldwide. For example, Taylor et al. (42) showed that bacterial chemoautotrophy, fueled by reduced sulfur species, supported an active secondary microbial food web in the redox transition zone of the Cariaco Basin. Depending on the season, dissolved inorganic carbon assimilation (27 to 159 mmol C m^{-2} day^{-1}) in this zone was equivalent to 10% to 333% of phytoplankton primary production. Madrid et al. (24) hypothesized that sulfide-oxidizing epsilon symbiont relative clones were responsible for the dark CO_2 fixation. Nitrate, manganese, and iron as potential electron acceptors were available for the epsilon symbiont relatives, but their reduction was not investigated. Jannasch et al. (17) isolated nine chemolithoautotrophic bacterial strains from the anoxic interface of the Black Sea. These isolates were unable to utilize nitrate, manganese, or iron oxides as electron acceptors. However, the authors postulated that this could have been due to toxic concentrations of the ions. In any case, since the oxidation of 1 mol of H_2S to sulfate requires 8 mol of Fe(III), the estimated iron fluxes could have accounted for only less than 0.1% of the measured H_2S oxidation rates in the suboxic zone of the Black Sea (17, 18).

The Baltic Sea itself is among the largest brackish basins of the world with periodically anoxic conditions in bottom waters. Analogously to the Black Sea, the Baltic Sea proper comprises a number of deep areas with anoxic bottom water, of which the Gotland Deep is the largest and the Landsort Deep, at 495 m, the deepest. In both deeps a stable halocline below 50 to 60 m separates the water column into the upper oxygenated layer and the underlying oxygen-deficient and anoxic/sulfidic layer (22, 28). The oxic-anoxic interfaces are generally characterized by high CO_2 dark fixation rates, which may correspond to up to 30% of surface primary production (8). The simultaneous occurrence of high denitrification rates (4, 34) led to the conclusion that chemolithoautotrophic oxidation of sulfur compounds coupled to nitrate reduction should play an important role in these pelagic redoxclines. Subsequently, an epsilonproteobacterium related to *Thiomicrospira denitrificans* was identified as an important chemolithoautotrophic sulfide oxidizer and nitrate reducer (16, 20). Additionally, there is first evidence that this bacterium was predominantly responsible for autotrophic denitrification processes at the Gotland Deep (3). However, for this site geochemical evidence also points to-

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wards the importance of redox processes related to manganese and iron transformations (28). Additionally, sulfide and ammonium, another well known electron donator for aerobic chemolithoautotrophs, generally occur together in this pelagic redoxcline. Altogether, the importance of different potential electron donors/acceptors for different chemolithoautotrophic microorganisms is still poorly known.

Thus, our main goal was to identify bacterial phylotypes of the Gotland Deep whose activity in terms of 16S rRNA synthesis could be stimulated, along with enhanced CO_2 fixation rates, upon the addition of other potential substrate combinations. Our second aim was to determine if the above-mentioned bacterium related to *T. denitrificans*, responsible for autotrophic nitrate reduction in the Gotland Deep, could also be a key player in autotrophic nitrate reduction in the Landsort Deep. This assumption was supported by the fact that phylogenetic relatives of *T. denitrificans* had been found in other marine oxic-anoxic interfaces, e.g., the Black Sea (44), the Cariaco Basin (24), or aphotic sulfidic springs (9, 10).

For this purpose, water samples of the two deeps were spiked by the addition of different substrate combinations. CO_2 fixation was analyzed and stimulated bacteria identified by molecular analysis as described previously (3, 20). This approach led to the successful identification of potential key chemolithoautotrophic organisms from two pelagic redoxclines of the central Baltic Sea.

MATERIALS AND METHODS

Sampling. Sampling was performed onboard the *RV Professor Albrecht Penck* during cruise 40/04/17 in the eastern Gotland Deep (Baltic Sea monitoring station 271; 57°19.2′N, 20°03′E) and in the Landsort Deep (station 284; 58°35′N, 18°14′E) (Fig. 1) in August 2004 under comparable physicochemical conditions.

Physicochemical structure of the oxic-anoxic interface. Chemical profiles of oxygen, hydrogen sulfide, ammonia, nitrite, and nitrate were determined as described elsewhere (14). Dissolved manganese and total manganese were analyzed onboard using the formaldoxime method (5). Briefly, 50-ml PE bottles were rinsed with deionized water and 20 ml 0.2-μm-filtered water sample. Subsequently, dissolved manganese was determined after filtration of 20 ml water sample into the bottle, mixed with 2 ml formaldoxime reagent, incubated for 60 min in the dark, and analyzed at 450 nm in a spectrophotometer (RF-1502; Shimadzu). Total manganese was determined accordingly from nonfiltered samples. Particulate manganese was sufficiently abundant at station 271 and could therefore be determined by subtraction of dissolved manganese from total manganese.

In situ CO_2 dark fixation. In situ CO_2 fixation rates were determined throughout the redoxcline according to the method of Steemann Nielsen (40). Briefly, incubations were carried out in 120-ml Winkler bottles to which 100 μCi [^{14}C]bicarbonate (specific activity, 53.0 mCi mmol^{-1}; Hartmann Analytic GmbH, Braunschweig, Germany) were added. Samples were incubated at in situ temperatures for 24 h in darkness, filtered on 0.2-μm-pore-size membrane filters, and exposed to HCl fumes for 20 min, and radioactivity was counted in a scintillation counter (Packard). Parallel samples immediately fixed with formalin (1.5% final concentration) before addition of [^{14}C]bicarbonate served as controls.

Stimulated in vitro CO_2 dark fixation. To assess the microbial diversity associated with in situ CO_2 dark fixation, we performed stimulation experiments. Sulfidic water samples were collected from 227-m-deep water at station 271 or from 85-m-deep water at station 284, using 5-liter Teflon-coated Go Flo bottles (General Oceanics) attached to a polyvinyl chloride-coated stainless steel conductivity-temperature-depth rosette, and then directly transferred, air bubble free, into plastic cans. Eventually, the samples were incubated at 4°C in a container with anoxic sulfide-free seawater. Due to diffusion processes, samples were free of sulfide and contained up to 15 μmol liter^{-1} oxygen after 4 days of incubation. Eventually, 630 ml or 120 ml (in the latter case together with [^{14}C]bicarbonate) was placed in transfusion bottles (Glasgerätebau Ochs, Bovenden, Germany) or Winkler bottles, respectively, and complemented by the addition of

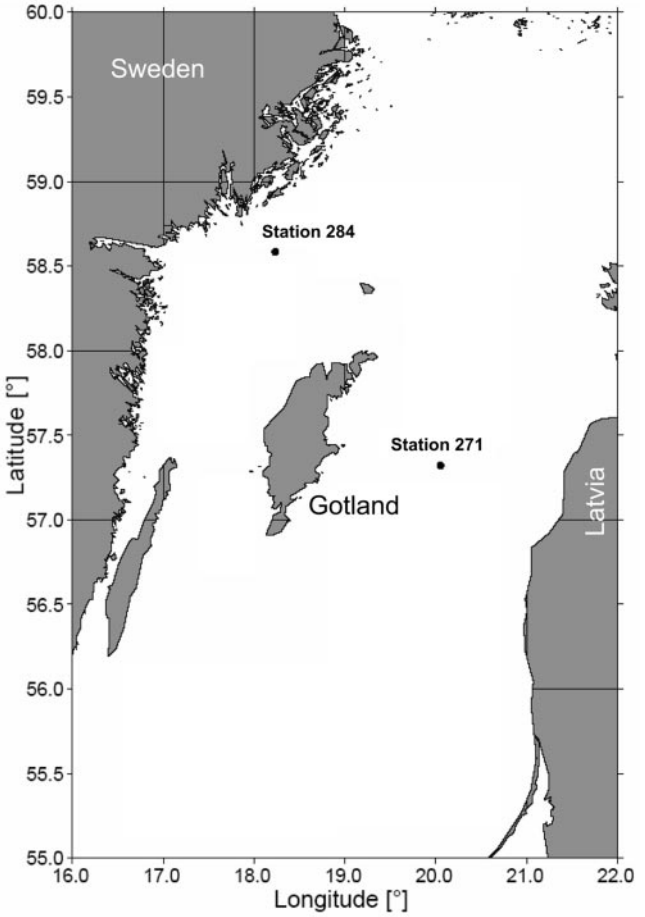

FIG. 1. Map of the central Baltic Sea and positions of sampling stations 271 and 284 (courtesy of Jan Donath, Leibniz-Institut für Ostseeforschung Warnemünde).

electron donor/acceptor pairs (Table 1) potentially supporting CO_2 fixation activity. The electron donor/acceptor combinations were added in three replicates as described previously (20) and incubated at 10°C for 72 h in the dark. All mixtures including nitrate or nitrite were prepared with Landsort water; the others were prepared with Gotland water. Potential heterotrophic CO_2 fixation was stimulated by the addition of glucose. Negative controls for all treatments were incubated at 0°C. After 0, 48, or 72 h of incubation, CO_2 dark fixation was measured as described before. In parallel, biomass of the enrichments was harvested by the filtration of 600 ml enrichment water on Durapore filters (0.2-μm pore size) and stored frozen (−80°C) for later analysis. The analysis of dissolved and particulate Mn or Fe phases during stimulation experiments was

TABLE 1. Electron donor/acceptor combinations used in the enrichment experiments[a]

Electron acceptor	Use[b] with the following electron donor:						
	Na_2S	$Na_2S_2O_3$	NH_4Cl	$MnCl_2 \cdot 4H_2O$	$FeCl_2$	CH_3OH	$C_6H_{12}O_6$[c]
MnO_2		×	×		×	×	×
$FeCl_3 \cdot 6H_2O$		×	×			×	×
KNO_3	×	×	×	×	×	×	×
KNO_2			×				

[a] The concentration of all components was 100 μmol liter^{-1}, except MnO_2, which was at 83.5 μmol liter^{-1}.
[b] ×, used in combination.
[c] Stimulation of heterotrophic CO_2 fixation.

based on previously described procedures (30), using a Perkin-Elmer AA spectrophotometer (models 3030 and ZL 4100 with Zeeman correction) in conjunction with an HGA 600 graphite furnace and an autosampler (model AS 60).

Stimulation and identification of microbial phylotypes. Nucleic acid extraction from frozen filters was performed for all thiosulfate-stimulated samples as described by Weinbauer et al. (46). Prior to reverse transcription-PCR, RNA extracts were purified from DNA by incubation with DNase I (Roche Diagnostics, Mannheim, Germany) for 60 min at 37°C, and their concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). To retrieve 16S ribosomal complementary DNA (rcDNA), 20 ng of template RNA was reverse transcribed at 42°C using the iScript cDNA synthesis kit (Bio-Rad). In addition to hexamers provided in the kit, the universal reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (21) was also supplied. Bacterial Com-primers (amplifying positions 519 to 926 [*Escherichia coli* numbering of 16S rRNA gene]) (36) were used for 16S rcDNA amplification. Thermocycling started with an initial denaturation for 5 min at 94°C. A total of 23 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C were followed by a final elongation step of 10 min at 72°C. Generation and purification of single-stranded DNA and single-strand-conformation polymorphism (SSCP) analysis were done as described by Schwieger and Tebbe (36).

The relative fractions of 16S rcDNA bands were determined by image analysis of silver stained SSCP gels using the GelScan software (BioSciTec). The relative fraction of bands was calculated by the determination of band areas.

Reamplification of individual bands excised from the SSCP gels was performed as described by Pöhler et al. (31). PCR products were purified using the MiniElute kit (QIAGEN) as described by the manufacturer and sequenced by SEQLAB (Göttingen, Germany). Forward and reverse sequences of all samples were checked for accuracy using the SeqMan software (DNAstar). Phylogenetic affiliations of the partial sequences were initially estimated using the program BLAST (1). Sequences were aligned and analyzed by employing the ARB software package (23). Sequences with greater than 98% similarity were grouped for phylogenetic analyses. Sequences for analysis were reduced to unambiguously alignable positions by using group-specific filters. An evolutionary distance dendrogram was constructed using maximum likelihood as well as the Jukes-Cantor correction and neighbor joining. Bootstrap analyses were performed for neighbor joining with 1,000 resamplings.

Validation of SSCP-based quantification by quantitative PCR (qPCR). An i-cycler IQ real-time system (Bio-Rad) was used to quantify the relative 16S rRNA concentration of an uncultured autotrophic nitrate-reducing *Helicobacteraceae* strain G138eps1 bacterium stimulated in the thiosulfate/nitrate enrichment after 72 h of incubation as described previously (16, 20). The PCR mixtures (25 μl) contained 1× iQSybrGreen Supermix (Bio-Rad), bacterial Com (900 nmol liter^{-1} each) or strain-specific OST 1 real-time primer system (OST 1F, 900 nmol liter^{-1}; OST 1R, 300 nmol liter^{-1}) (16, 20), and 3 ng of nucleic acids. An initial denaturing step at 95°C for 3 min was followed by 40 cycles of 94°C for 30 s, 59°C for 40 s, and 72°C (measuring step) for 50 s. Potential development of primer dimers was determined by a melting point analysis in a range from 40 to 94°C. Each measurement was performed in three replicates. Relative standards were prepared using serially diluted (1:10) nucleic acids again isolated from the thiosulfate/nitrate enrichment. Each sample measured was calibrated by its own respective Com and OST standard. Due to potentially lower PCR efficiencies of nondiluted cDNA extracts, each sample to be measured was always diluted 1:10. Calibration curves as well as quantifications were generated by the i-cycler IQ real-time system software. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or cycle threshold value. Only data resulting from measurements with comparable Com- and OST-specific PCR efficiencies were used for further calculations.

Estimation of cell concentration and doubling time. The measured carbon fixation during the stimulation experiments was assumed to represent the biomass of chemolithoautotrophic bacteria build up by exponential growth during the incubation time. Given that only new bacterial biomass was labeled by ^{14}C, the biomass at time $t = 0$ h (X_0) had to be calculated. Thus, the total biomass of chemolithoautotrophic bacteria at time t_1 was equal to the new biomass X_1 plus the unknown X_0.

Normal bacterial growth was described by the equation $X_t = X_0 \times e^{\mu \times t}$. The biomass built after addition of $^{14}CO_2$ was measured. The unlabeled biomass of the bacterial fraction taking up $^{14}CO_2$ was unknown (called X_0). Thus, the following equation was used for the incubation periods: $X_t + X_0 = X_0 \times e^{\mu \times t}$. This equation was solved for μ, to give $\mu = [\ln(X_t + X_0) - \ln X_0]/t$.

For the two incubation periods the following equation results when μ is assumed to be constant during the experiment: $[\ln(X_1 + X_0) - \ln X_0]/t_1 = [\ln(X_2 + X_0) - \ln X_0]/t_2$. Based on the new biomasses X_1 and X_2 determined at points t_1 and t_2, respectively, X_0 was calculated by iteration: $t_1 \times [\ln(X_2 + X_0) - \ln X_0] - t_2 \times [\ln(X_1 + X_0) - \ln X_0] = 0$.

The initial biomass X_0 was transformed to cell concentration N_0 by assuming an individual cell biomass of 20 fg C (12). The mean growth rate was calculated using the equation for μ above and transformed to doubling time.

Nucleotide sequence accession numbers. Sequences have been deposited in the EMBL nucleotide sequence database under accession numbers AJ937823 to AJ937832.

RESULTS

Physicochemical structure of the oxic-anoxic interface. Physical and chemical parameters of stations 271 and 284 are shown in Fig. 2. Due to the invasion of oxygenated North Sea water in 2003, the pelagic redoxcline at station 271 was newly reestablished approximately 20 m above the sediment (Fig. 2A). Below the halocline at a depth of 60 m, oxygen decreased sharply to concentrations of below 50 μmol liter^{-1}, reaching values of less than 20 μmol liter^{-1} at depths of around 200 m. From this depth until the redoxcline, oxygen decreased continuously. Nitrate decreased sharply from 225 to 232 m. Dissolved manganese, ammonium, and sulfide increased strongly around and below the redoxcline. Particulate manganese showed its maximum well above the redoxcline at around 210 m. The redoxcline at station 284 was established at a depth of approximately 70 to 80 m (Fig. 2B). With the exception that manganese concentrations in a range from 0.17 to 0.21 μmol liter^{-1} at station 284 were approximately 100-fold lower than those at station 271, the chemical profile displayed similar characteristics.

CO_2 fixation rates. At station 271, the highest in situ CO_2 fixation rates of 0.7 μmol liter^{-1} day^{-1} were observed in the sulfidic zone directly below the redoxcline at a depth of approximately 227 m (Fig. 2A). In the enrichment experiments, the highest CO_2 dark fixation rates were detected when thiosulfate was used as an electron donor. The combinations $Na_2S_2O_3$/Fe(III) and $Na_2S_2O_3$/Mn(IV), and even $Na_2S_2O_3$ alone, resulted in high CO_2 fixation rates, but no significant reduction of the respective electron acceptors could be measured (Table 2). The combinations NH_4^+/Mn(IV) and methanol/Mn(IV) yielded no detectable CO_2 dark fixation. Thus, the bacterial community was not further analyzed.

At station 284, in situ CO_2 fixation rates reached approximately 1.4 μmol liter^{-1} day^{-1} in the sulfidic zone below the redoxcline beginning at a depth of approximately 85 m (Fig. 2B). No decrease of in situ CO_2 fixation rates was observed down to the last investigated depth of 96 m. Highest CO_2 fixation as well as nitrate reduction rates were determined for the $Na_2S_2O_3/NO_3^-$ enrichment. Other electron donators, even sulfide, yielded significantly less or, in the case of Fe(II)/NO_3^- and NH_4^+/NO_2^-, no detectable CO_2 dark fixation (Table 2).

Heterotrophic CO_2 dark fixation. Artificially stimulated heterotrophic CO_2 dark fixation reached 0.15 to 0.17 μmol liter^{-1} after 48 h at both stations. It increased exponentially, reaching 0.84 μmol liter^{-1} day^{-1}, from 48 to 72 h. In the cases of Fe(III) and NO_3^-, this was accompanied by a reduction of these electron acceptors (Table 2).

Stimulated microbial communities. SSCP profiling revealed distinct community changes in the enrichment assays (Fig. 3). Phylogenetic analysis of SSCP band sequences revealed 16S rRNA similarities to already described species or clones in the

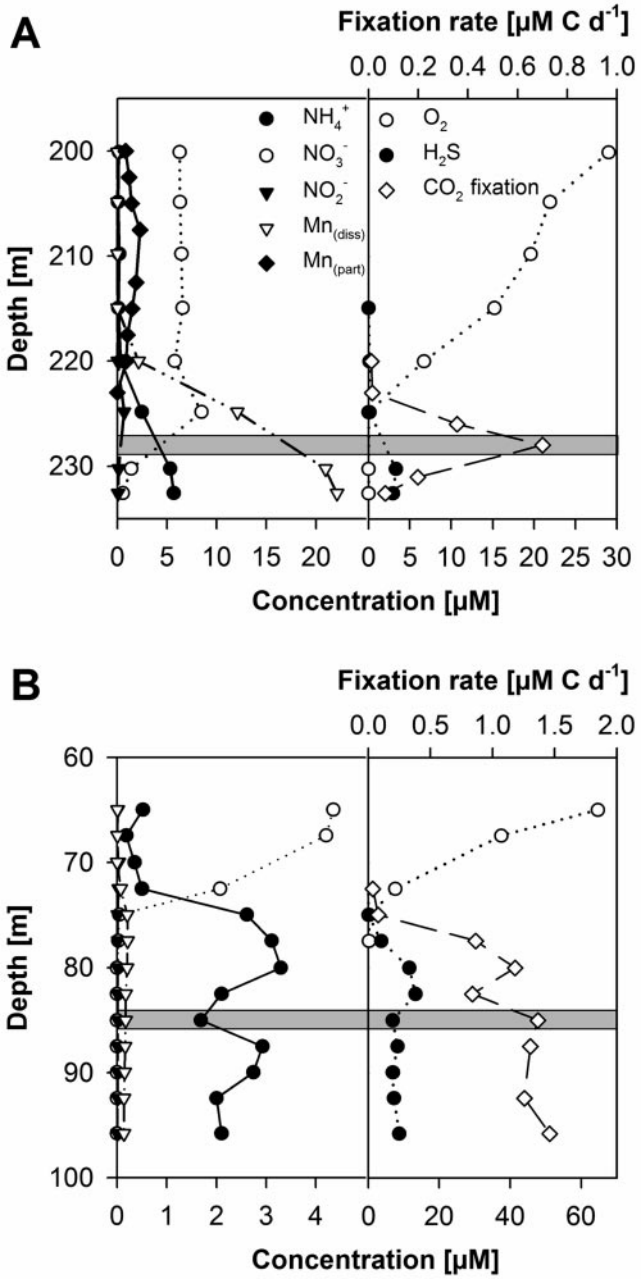

FIG. 2. Depth profiles of NH_4^+, NO_3^-, NO_2^-, and particulate and dissolved Mn (left), as well as O_2, H_2S, and $^{14}CO_2$ fixation (right), throughout the oxic-anoxic interface of Gotland Deep station 271 (A) and Landsort Deep station 284 (B) in August 2004. The shaded area indicates the CO_2 fixation zone which was sampled for subsequent stimulation experiments.

range from 94 to 100% (Fig. 3; Table 3). With bacteria closely related or identical to the aerobically sulfide-oxidizing *Thiomicrospira psychrophila* and *Maorithyas hadalis* gill symbiont as well as the uncultured autotrophic, denitrifying *Helicobacteraceae* strain G138eps1 (Table 4), the highest diversity of stimulated potential chemolithoautotrophic bacteria from station 271 was observed in the $Na_2S_2O_3$ enrichment after 48 h of incubation (Table 4). *T. psychrophila* and relatives of the uncultured *Helicobacteraceae* were stimulated in the $Na_2S_2O_3$/Fe(III) and $Na_2S_2O_3$/Mn(IV) enrichments during the whole experiment. Three organisms related to *Pseudomonas stutzeri* or fish-pathogenic *Pseudomonas anguilliseptica* and uncultured CFB group bacterium MERTZ_2CM_22 were stimulated in the $Na_2S_2O_3$/Fe(III) enrichment (Fig. 3). Relatives of uncultured epsilonproteobacterium clone Nubeena319 and again the uncultured CFB group bacterium were stimulated in the $Na_2S_2O_3$ and $Na_2S_2O_3$/Mn(IV) enrichments (Table 4; Fig. 3 and 4).

With relative 16S rRNA quantities of 51% (after 48 h of incubation) and 35% (after 72 h), the $Na_2S_2O_3/NO_3^-$ enrichments were exclusively dominated by the autotrophic uncultured *Helicobacteraceae* strain G138eps1 (Table 4; Fig. 3). For a validation of this SSCP quantification approach, the relative 16S rRNA concentration after 72 h of incubation was additionally determined by qPCR as described previously for this specific organism (20). With a PCR efficiency of 101% and a correlation coefficient of the regression line of 0.997, its relative concentration was determined to be 32% ± 2.3%.

Estimation of doubling time and cell concentration. Calculated bacterial cell doubling times from the two CO_2 fixation measurements in all experiments using thiosulfate as an electron donor were in a range between 12 and 18 h (Table 5). Their initial cell numbers were calculated to be between 0.9×10^5 and 5.6×10^5 ml^{-1}. Cell numbers for the thiosulfate/Mn(IV) enrichment could not be calculated, because CO_2 fixation rates were not increasing exponentially with this treatment (Table 2).

DISCUSSION

CO_2 fixation rates. High dark carbon fixation had already been observed at redoxclines of different aquatic areas (6, 24, 39, 47). Typically, ammonia oxidation and oxidation of reduced sulfur compounds were related to it using oxygen as electron acceptor. In the redoxcline of the central Baltic Sea, NH_4^+ was generally present in concentrations similar to those for sulfide (Fig. 2A). However, reduced sulfur compounds and not ammonium oxidation seemed to play the major role for the autotrophic CO_2 fixation (Table 2). Enoksson (11) measured nitrification rates not higher than 280 nmol liter^{-1} per day. With a commonly used factor for conversion to carbon fixation of 0.12 (2) and also a twofold underestimation, ammonia oxidation could have been responsible for less than 0.1 μmol liter^{-1} carbon dioxide fixation per day. Thus, ammonia oxidation should not be the main process responsible for the observed high CO_2 fixation rates.

Moreover, it is interesting to note that CO_2 dark fixation was less stimulated by the addition of sulfide than by thiosulfate (Table 2). This could be due to inhibitory effects of sulfide, because as little as 300 μmol liter^{-1} H_2S resulted in almost total inhibition of nitrous oxide reduction by denitrifying bacteria (38).

Due to the sampling procedure for in situ analyses onboard, small concentrations of oxygen possibly diffused into the samples. This potentially forced autotrophic sulfide oxidation by this artificially supplied oxygen and could have influenced the location of the in situ CO_2 fixation maximum. However, high-

TABLE 2. CO_2 fixation and oxidation/reduction rates after 48 and 72 hours of incubation with different electron donator/electron acceptor combinations

Station and electron donor/electron acceptor	Mean (μmol liter^{-1})[c]								
	CO_2 fixation			Oxidation of electron donor			Reduction of electron acceptor		
	0–48 h	48–72 h	Daily rate	0–48 h	48–72 h	Daily rate	0–48 h	48–72 h	Daily rate
Station 271									
Methanol/Fe(III)	0.14 (0.04)	−0.05 (0.00)	0.03	ND[a]	ND	ND	−9.1 (4.3)	9.4 (42.3)	0.1
Fe(II)/Mn(IV)	0.15 (0.05)	−0.10 (0.06)	0.02	17.8 (4.3)	12.4 (1.6)	10.1	30.6 (0.9)	19.7 (4.5)	16.8
NH_4^+/Fe(III)	0.33 (0.33)	−0.28 (0.03)	0.02	−53.7 (11.8)	68.0 (14.3)	7.2	ND	ND	ND
$Na_2S_2O_3$/Fe(III)	2.08 (0.03)	5.65 (0.56)	2.58	ND	ND	ND	1.1 (0.9)	−9.1 (1.2)	−2.7
$Na_2S_2O_3$/none	2.43 (0.04)	8.91 (0.20)	3.78	ND	ND	ND	ND[b]	ND	ND
$Na_2S_2O_3$/Mn(IV)	2.45 (0.01)	1.68 (0.09)	1.38	ND	ND	ND	11.3 (1.3)	−11.3 (0.0)	0.0
Glucose/Mn(IV)	0.15 (0.11)	0.84 (0.01)	0.33	ND	ND	ND	−0.7 (8.1)	−1.2 (2.2)	−0.6
Glucose/Fe(III)	0.16 (0.06)	0.70 (0.29)	0.29	ND	ND	ND	−6.9	4.4 (2.8)	−0.8
Station 284									
$Na_2S_2O_3/NO_3^-$	4.93 (0.25)	9.68 (0.41)	4.87	ND	ND	ND	27.2 (7.3)	33.5 (9.0)	20.2
Na_2S/NO_3^-	0.93 (0.64)	−0.21 (0.17)	0.24	42.4 (11.2)	−19.7 (5.1)	7.6	ND	ND	ND
NH_4^+/NO_3^-	0.04 (0.01)	0.10 (0.09)	0.05	4.9 (0.7)	−35.6 (4.8)	−10.2	ND	ND	ND
Methanol/NO_3^-	0.06 (0.01)	0.02 (0.01)	0.03	ND	ND	ND	21.0 (8.2)	−18.7 (7.3)	0.8
Mn(II)/NO_3^-	0.07 (0.00)	0.00 (0.00)	0.02	−3.4 (4.8)	3.4 (0.0)	0.0	ND	ND	ND
Glucose/NO_3^-	0.17 (0.09)	0.22 (0.03)	0.13	ND	ND	ND	−10.7 (2.9)	8.3 (2.2)	−0.8

[a] ND, not determined.
[b] 0.01 ml liter^{-1} H_2S detected.
[c] Values in parentheses are standard deviations in micromoles per liter.

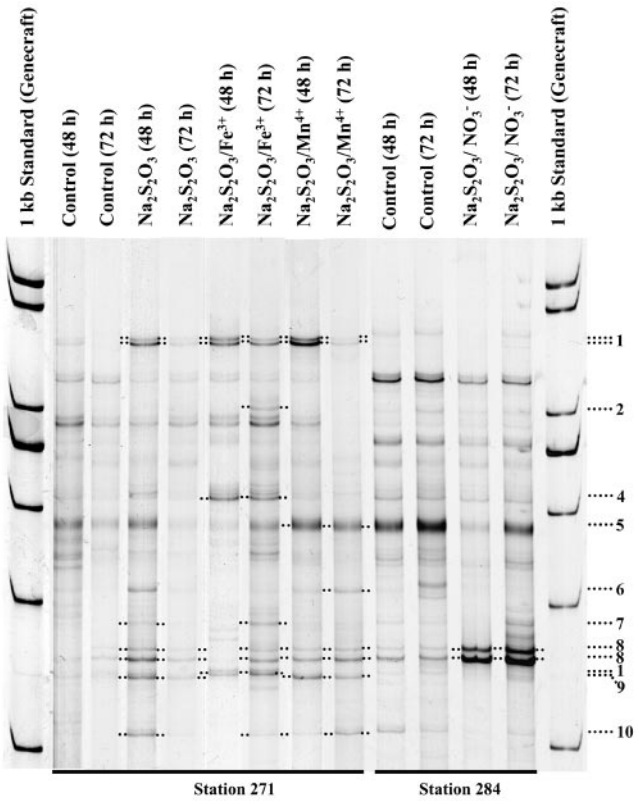

FIG. 3. 16S rcDNA SSCP analysis of thiosulfate stimulation experiments using different electron acceptors. The original nonstimulated seawater sample served as a control. Stimulation time is given in parentheses. Numbers on the right refer to excised and sequenced bands (Table 3).

est rates of dark $H^{14}CO_3^-$ fixation in the Mariager Fjord were also observed at the lower border of the redoxcline (47).

In general, it has been suggested that 3 to 8% of the organic carbon in some heterotrophic bacteria originates from CO_2 incorporated by carboxylation reactions (35). However, it can be equally important as autotrophic CO_2 fixation (25) and could mask the autotrophic $^{14}CO_2$ fixation signal, especially in habitats with high input of organic material (47). If heterotrophic CO_2 fixation had occurred in situ, this activity should be detectable as background in all experiments. Therefore, we assume that the lowest measured CO_2 fixation due to in situ anaplerotic carbon fixation accounted for less than 0.1 μmol liter^{-1} within 48 h. Even artificially stimulated heterotrophic CO_2 dark fixation reached only 0.15 to 0.17 μM after 48 h. Higher rates (up to 0.84 μmol liter^{-1} day^{-1}) were measured only after 72 h and in the case of Fe(III) and NO_3^- were accompanied by a reduction of these electron acceptors (Table 2). Altogether, we conclude that heterotrophic CO_2 dark fixation should have been negligible for the time of our sampling for the two stations. CO_2 dark fixation should have been predominantly due to the activity of sulfide-oxidizing chemolithoautotrophic bacteria. However, heterotrophic Fe(III) reduction could potentially play a role in pelagic redoxclines of the Gotland Deep.

The combinations $Na_2S_2O_3$/Fe(III) and $Na_2S_2O_3$/Mn(IV) and even $Na_2S_2O_3$ alone resulted in high CO_2 fixation rates in Gotland Deep water, but no significant reduction of the respective electron acceptors was detectable (Table 2), even assuming that soluble pools of organically complexed Fe(III) and Mn(IV) could have increased the amount of reduced substances artificially. In general, denitrification takes place already at low oxygen concentrations of approximately 10 to 15 μmol liter^{-1} (34). This was also demonstrated for our experiments, where autotrophic nitrate reduction was stimulated with a daily mean CO_2 dark fixation of 2.5 μmol liter^{-1} during

TABLE 3. 16S rRNA sequence designations, sequence origins, nucleotide sequence accession numbers, sequence lengths, and closest phylogenetic neighbors of investigated sequences

Sequence designation (SSCP band)	Sequence origin (enrichment)	Nucleotide sequence accession number	Sequence length (bp)	Closest phylogenetic neighbor	Sequence similarity (%)[a]
1	Thiosulfate	AJ937823	345	*Thiomicrospira psychrophila* SVAL-D	99
2	Thiosulfate/iron	AJ937824	347	*Pseudomonas stutzeri*	99
4	Thiosulfate/iron	AJ937826	299	*Pseudomonas* sp. strain He	99
5	Thiosulfate/manganese	AJ937827	331	*Thalassolituus oleivorans*	98
6	Thiosulfate/manganese	AJ937828	300	*Maorithyas hadalis* gill thioautotrophic symbiont	99
7	Thiosulfate/manganese	AJ937829	329	Uncultured *Helicobacteraceae* strain G138eps1	96
8	Thiosulfate	AJ937830	349	Uncultured *Helicobacteraceae* strain G138eps1	100
9	Thiosulfate	AJ937831	350	Uncultured epsilonproteobacterium clone Nubeena319	100
10	Thiosulfate	AJ937832	341	Uncultured CFB group bacterium MERTZ_2CM_22	94

[a] Determined by the program BLAST.

the first 48 h, followed by an increase up to 9.7 μmol liter^{-1} day^{-1} (Table 2). However, similar oxygen conditions did not result in analyzable Fe(III) or Mn(IV) reduction, leading to the conclusion that solely oxygen could have been reduced instead. Thus, potential autotrophic Fe(III)/Mn(IV) reduction might be restricted to strictly anaerobic areas of the pelagic redoxcline, even at high concentrations of their particulate oxides (Fig. 2A). In the Gotland Deep, respective areas could be reached with a flux of settling Mn oxides (28) and probably by Fe oxides. However, the importance of this mechanism has to be further analyzed.

The combination Fe(II)/Mn(IV) showed iron oxidation rates of 10 μmol liter^{-1} day^{-1} and manganese reduction rates of 17 μmol liter^{-1} day^{-1} (Table 2). However, it should be noted that Fe(II) readily reduces Mn(IV) in a purely chemical redox reaction, and these abiotic chemical transformations could have been inhibited in the control experiments at lower temperatures.

Stimulated microbial communities. In Gotland Deep water, a close phylogenetic relative of the aerobically sulfide-oxidizing and chemolithoautotrophic *Thiomicrospira psychrophila* (19) was stimulated in all thiosulfate enrichments. However, it is also interesting to note that a member of the *Pseudomonas anguilliseptica* group (Fig. 4) was stimulated in the $Na_2S_2O_3$/Fe(III) enrichment (Table 4). *P. anguilliseptica* causes red spot disease in several fishes and was originally isolated from pond-cultured *Anguilla japonica* eels (45). It could be speculated that iron-rich, low-oxygen marine or brackish water habitats could be natural reservoirs for these bacteria.

As already mentioned, the $Na_2S_2O_3/NO_3^-$ enrichment with Landsort Deep water was dominated solely by a potential autotrophic denitrifying bacterium with sequences 100% identical to uncultured *Helicobacteraceae* 16S rRNA sequences (Tables 3 and 4; Fig. 4) and related to the anaerobic chemolithoautotrophic *Thiomicrospira denitrificans* (43). This dominance was identified by 16S rcDNA fingerprinting, which, due to the PCR bias, can only be accepted to be "semiquantitative." However, we were able to validate these quantifications by qPCR, because a real-time protocol for a relative quantification of the uncultured *Helicobacteraceae* strain G138eps1 was already established earlier (20). With nearly identical 16S rRNA abundances of 35% (analyzed by SSCP) (Table 4) and 32% (analyzed by qPCR), the SSCP approach seemed to be appropriate for quantifications in this case.

CO_2 dark fixation was almost exclusively due to the activity of chemolithoautotrophic bacteria. Their calculated bacterial cell doubling in the range between 12 and 18 h (Table 5) was comparable to that of heterotrophic marine bacterial populations (15). Cell numbers reached 5.6×10^5 ml^{-1} for the autotrophic denitrifying *Helicobacteraceae* strain. Although this is

TABLE 4. Identification of substrate-stimulated 16S rRNA phylotypes according to their relative rRNA abundances in SSCP gels

16S rcDNA SSCP band no.	Relative abundance (%)[a] with the indicated electron donor/electron acceptor combination											
	Station 271								Station 284			
	Control		$Na_2S_2O_3$/none		$Na_2S_2O_3$/Fe(III)		$Na_2S_2O_3$/Mn(IV)		Control		$Na_2S_2O_3/NO_3^-$	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
1[b]	7.6	0	**14**	**12.8**	**28.7**	**16.6**	**31.0**	**8.2**	0	0	0	0
2	0	0	0	0	0	**3.5**	0	0	0	3.9	0	3.1
4	0	0	0	0	**25.0**	**11.1**	0	0	5.2	3.3	4.7	3.5
5	20.4	22.5	18.6	19.2	13.7	12.2	**32.2**	**29.8**	26.9	30.2	11.5	17.6
6[b]	0	0	**8.7**	0	0	0	0	**9.2**	0	0	0	0
7[b]	5.7	0	5.5	0	0	3.9	0	0	0	0	0	0
8[b]	3.9	14.0	**17.0**	15.8	**6.5**	11.1	**13.0**	**19.9**	7.9	6.3	**50.9**	**35.0**
9	4.4	0	**11.5**	**9.2**	0	0	**12.7**	**10.3**	0	0	0	0
10	0	0	**8.4**	0	0	**3.6**	0	**10.2**	4.4	2.6	0	1.5

[a] Relative abundance in the nonstimulated control and after 48 and 72 hours of stimulation with thiosulfate and different electron acceptors. Organisms were considered stimulated if their abundance increased at least by 30% compared to the nonstimulated control (indicated in boldface).
[b] Potentially chemolithoautotrophic bacterium.

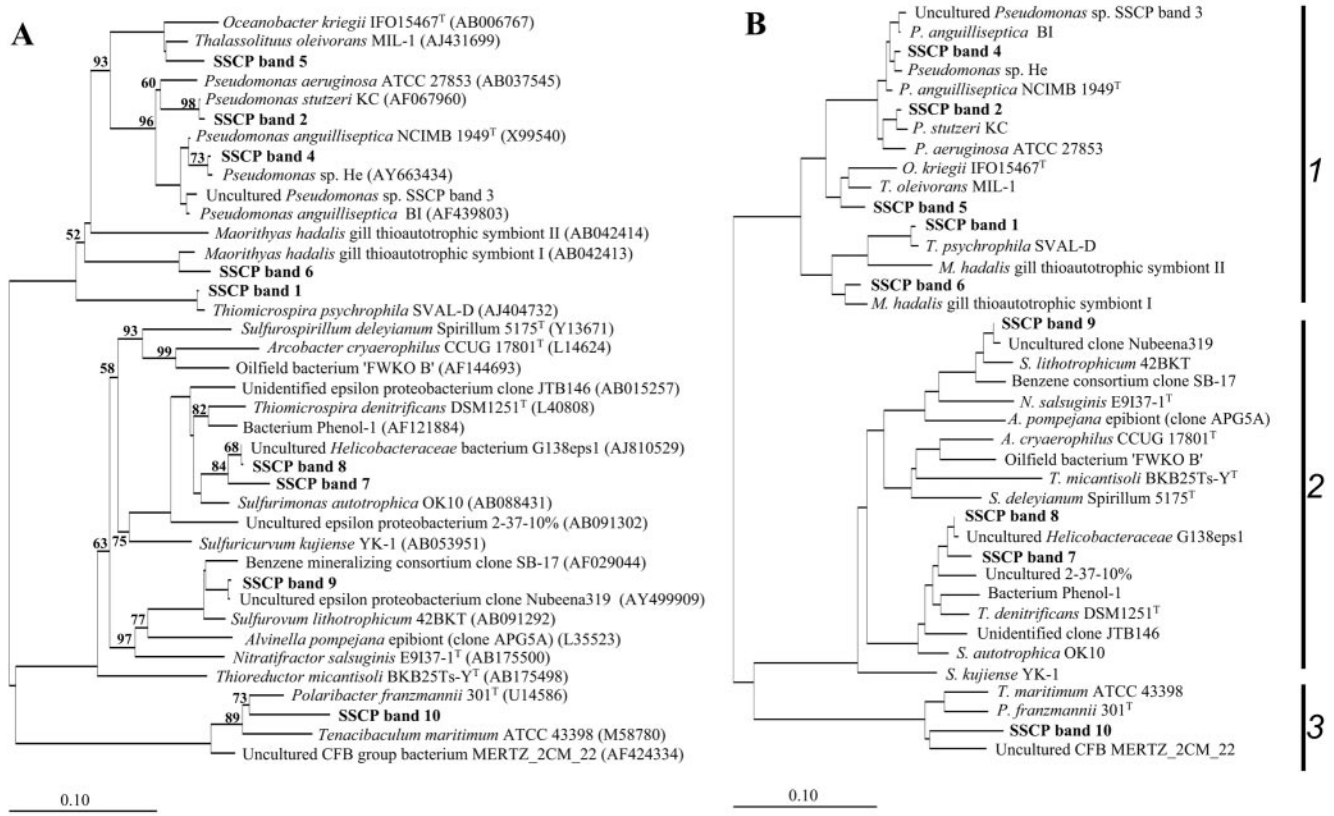

FIG. 4. Unrooted trees showing phylogenetic relationships of the stimulated bacteria and their closest phylogenetic relatives. (A) Tree reconstructed using the neighbor-joining method and based on a comparison of approximately 300 nucleotides. Bootstrap values, expressed as a percentage of 1,000 replications, are given at branching points. Only those above 50% are shown. Database accession numbers are given in parentheses. (B) Basic tree reconstructed using the maximum-likelihood method and based on a comparison of approximately 1,400 nucleotides. Eventually, partial SSCP sequences were individually imported using ARB Parsimony. For both tree reconstruction methods, 42 members of the *Verrucomicrobiaceae* were used as an outgroup. Bar, 10 substitutions per 100 nucleotides. 1, *Gammaproteobacteria*; 2, *Epsilonproteobacteria*; 3, *Bacteroidetes*. More detailed information about the investigated sequences is given in Table 3.

approximately three times higher than earlier estimated concentrations for this bacterium determined by strain-specific quantitative PCR for station 271, it is surprisingly similar considering that two different and separated habitats, sampling times, and calculation methods were bases for our calculations (20). This demonstrated the reproducibility of our approach but also the constancy of pelagic redoxcline assemblages in the Baltic Sea. For samples of Gotland Deep water from 1998, the uncultured *Helicobacteraceae* strain was proposed to be a key player for autotrophic denitrification (3). With both having two bands of identical sequence, which seemed to be characteristic for this bacterium, the 16S rcDNA SSCP fingerprints from 1998 and 2004 were very comparable (Fig. 3) (see Fig. 1 in reference 16). This underlined the stability of autotrophic nitrate-reducing assemblages in pelagic redoxclines of the Baltic Sea. However, phylogenetic relatives of this epsilonproteobacterium have also been detected in other, similar habitats worldwide (9, 10, 24, 44), indicating a general importance of this epsilonproteobacterium or its phylogenetic relatives for chemolithoautotrophic CO_2 fixation in pelagic redoxclines. In our experiments this phylotype was also independently stimulated in the $Na_2S_2O_3$ and $Na_2S_2O_3$/Mn(IV) enrichments (Table 4), possibly indicating broader physiological capacities of this organism. Physiological versatilities have already been reported for other chemolithoautotrophic bacterial groups. For example, anaerobic respiration using Fe(III) or S^0 as an electron acceptor and H_2 or S^0 as an electron donor served as a primary energy source of the well-known chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* (29). Pronk et al. (32, 33) and Das et al. (7) showed that this bacterium also grew with the oxidation of S^0 by Fe(III) under oxygen-limited and acidophilic conditions. To our knowledge, this has not been found for marine environments. However, several chemolithoautotrophic and versatile *Epsilonproteobacteria* have been described recently. For example, oxygen could serve as an alternative electron acceptor for denitrifying *Nitratiruptor tergarcus*

TABLE 5. Doubling time and estimated initial cell concentration of chemolithoautotrophic bacteria in different stimulation experiments with thiosulfate as the electron donator

Cell characteristic	Value with the following electron donor/electron acceptor combination:		
	$Na_2S_2O_3$/none	$Na_2S_2O_3$/Fe(III)	$Na_2S_2O_3/NO_3^-$
Doubling time (h)	12.0	14.4	18.2
Cell concn (N_0)	0.9×10^5	1.3×10^5	5.6×10^5

and *Nitratifractor salsuginis* (27). Strictly anaerobic *Thioreductor micantisoli* was able to oxidize hydrogen and reduce elemental sulfur or nitrate (26). Takai et al. (41) described several new strains that were able to oxidize hydrogen or reduced sulfur compounds and to reduce O_2, nitrate, or elemental sulfur. All of these strains were mesophilic to thermophilic; however, sulfide-oxidizing microaerophilic *Thiomicrospira* sp. strain CVO was also able to grow at lower temperatures and also might utilize limiting amounts of oxygen or nitrate (13).

In conclusion, our data indicated that at pelagic oxic/anoxic interfaces of the central Baltic Sea, chemolithoautotrophic CO_2 dark fixation was due to nitrate and oxygen but not to Fe(III)/Mn(IV) reduction in combination with reduced sulfur compounds. An epsilonproteobacterium appeared to be a key organism for autotrophic nitrate reduction in different pelagic redoxclines of the Baltic Sea. Ammonium did not stimulate CO_2 dark fixation significantly. Thus, the relevance of a potential autotrophic Fe(III)/Mn(IV) reduction for the (bio-)geochemical cycling and fluxes of these ions has to be investigated further, especially under strictly anaerobic conditions. Moreover, our findings support the well-known fact that phylogenetic identification of microorganisms alone does not necessarily give insight into their functional role in vivo. This underlines the importance and usefulness of our approach of combining the identification of stimulated bacterial phylotypes with the analysis of CO_2 fixation rates upon the addition of potential substrate combinations in order to investigate their role in biogeochemical cycles in redoxclines of the Baltic Sea.

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