Epsilonproteobacteria Represent the Major Portion of Chemoautotrophic Bacteria in Sulfidic Waters of Pelagic Redoxclines of the Baltic and Black Seas

Jana Grote, Günter Jost, Matthias Labrenz, Gerhard J. Herndl, and Klaus Jürgens

Leibniz Institute for Baltic Sea Research, Section Biology, Seestrasse 15, 18119 Rostock-Warnemuende, Germany, and Department of Biological Oceanography, Royal Netherlands Institute for Sea Research, 1790 AB Den Burg, The Netherlands

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Recent studies have indicated that chemoautotrophic Epsilonproteobacteria might play an important role, especially as anaerobic or microaerophilic dark CO2-fixing organisms, in marine pelagic redoxclines. However, knowledge of their distribution and abundance as actively CO2-fixing microorganisms in pelagic redoxclines is still deficient. We determined the contribution of Epsilonproteobacteria to dark CO2 fixation in the sulfidic areas of central Baltic Sea and Black Sea redoxclines by combining catalyzed reporter deposition-fluorescence in situ hybridization with microautoradiography using [14C]bicarbonate and compared it to the total prokaryotic chemoautotrophic activity. In absolute numbers, up to \(3 \times 10^5\) 14CO2-fixing prokaryotic cells ml\(^{-1}\) were enumerated in the redoxcline of the central Baltic Sea and up to \(9 \times 10^4\) 14CO2-fixing cells ml\(^{-1}\) were enumerated in the Black Sea redoxcline, corresponding to 29% and 12%, respectively, of total cell abundance. 14CO2-incorporating cells belonged exclusively to the domain Bacteria. Among these, members of the Epsilonproteobacteria were approximately 70% of the cells in the central Baltic Sea and up to 100% in the Black Sea. For the Baltic Sea, the Sulfurimonas subgroup GD17, previously assumed to be involved in autotrophic denitrification, was the most dominant CO2-fixing group. In conclusion, Epsilonproteobacteria were found to be mainly responsible for chemoautotrophic activity in the dark CO2 fixation maxima of the Black Sea and central Baltic Sea redoxclines. These Epsilonproteobacteria might be relevant in similar habitats of the world’s oceans, where high dark CO2 fixation rates have been measured.

Pelagic redoxclines represent the transition zones between the oxic and anoxic realms. Extensive pelagic redoxclines are reported for the Black Sea (18, 34, 36), the Cariaco Basin (40, 42), the Framvaren Fjord (27), the Mariager Fjord (45), the Baltic Sea (19), and freshwater lakes (6, 9, 14) and are often characterized by high dark CO2 fixation rates. Characteristically, the peak of dark CO2 fixation within the water column is often located below the chemocline, which we define as the shallowest appearance of sulfide (13). Epsilonproteobacteria have already been suggested to be involved in chemooautotrophic production at several marine pelagic redoxclines (21, 23, 26, 43), and their prevalence at oxic-anoxic transition zones, especially in areas with previously detected high chemooautotrophic activity, has been demonstrated for the Black Sea and the Cariaco Basin (23, 24, 26, 43). Whether Epsilonproteobacteria are indeed responsible for chemooautotrophic production, however, has not been shown for any of these habitats.

For the Baltic Sea, Jost et al. (19) determined the abundance of chemooautotrophic cells in waters below the chemocline by combining dark CO2 fixation measurements and flow-cytometric cell sorting. Two main prokaryotic cell clusters responsible for total dark CO2 fixation were detected, and 20 to 40% of the total prokaryotic community was estimated to be chemooautotrophic, but the phylogenetic identity of these clusters remained unknown. Recently, Glaubitz et al. (10) determined the diversity of chemooautotrophs using stable-isotope probing (rRNA-SIP) combined with fingerprint techniques in samples from the dark CO2 fixation maximum in the central Baltic Sea. Autotrophic activity of Gammaproteobacteria and Epsilonproteobacteria, mainly represented by the Sulfurimonas subgroup GD17, which previously has been found to be abundant in Baltic Sea redoxclines (4, 13, 21), was detected. However, these studies did not address the quantitative impact of Epsilonproteobacteria for chemooautotrophic activity in the central Baltic Sea. Consequently, the objective of the present work was to determine the contribution of Epsilonproteobacteria to dark CO2 fixation in sulfidic areas of marine redoxclines of the central Baltic Sea and the Black Sea.

For identification and quantification of metabolically active cells in environmental samples, the combination of fluorescence in situ hybridization (FISH) and microautoradiography has often been used (7, 12, 22). Here we used the more sensitive catalyzed reporter deposition (CARD)FISH protocol combined with microautoradiography (MICRO-CARD-FISH) to assess the specific uptake of radiolabeled bicarbonate by prokaryotic cells (41). Our results demonstrate, for the first time, with special emphasis on Epsilonproteobacteria, the quantitative distribution of CO2-fixing cells in the sulfidic areas of two marine redoxclines.

MATERIALS AND METHODS

Sampling. The samples were obtained from the central Baltic Sea during a research cruise onboard the RV Professor Albrecht Penck in April 2007 (station...
Water samples from the redoxclines were collected with free-flow bottles (Hydrolab) attached to a conductivity, temperature, and depth rosette (SBE 911+; Seabird). Concentrations of inorganic nutrients, oxygen, and hydrogen sulfide were analyzed as described by Grasshoff et al. (11).

Microautoradiographic incubations and dark CO₂ fixation measurements. Incubations for MICRO-CARD-FISH and the determination of dark CO₂ fixation rates started within one hour after collecting the samples from the free-flow bottles. Special care was taken to avoid oxygen contamination during sample collection. Therefore, samples were directly filled from free-flow bottles into the test tubes with an overflow of at least five tube volumes. In the Baltic Sea, samples were taken from three depths, and in the Black Sea, from six depths. One hundred µl of [¹⁴C]bicarbonate in anoxic solution (specific activity, 53.0 mCi mmol⁻¹; Hartmann Analytic GmbH, Braunschweig, Germany) was added to 9-ml glass test tubes containing the water samples. Thereafter, the tubes were sealed without headspace with glass stoppers. Controls were fixed with paraformaldehyde (2% final concentration) before the addition of the radiolabeled solution. After incubation at in situ temperatures (4°C for the Baltic Sea samples and 8°C for the Black Sea samples) for 24 h in the dark, 1-ml subsamples were filtered through 0.2-µm-pore-size cellulose nitrate filters (diameter, 25 mm; Purabind 02; Whatman) and exposed to HCl fumes, and the radioactivity of the individual filters was counted in a liquid scintillation counter (Packard). The activity ranged between 99 and 6,707 dpm ml⁻¹ of filtered sample. For MICRO-CARD-FISH, the remaining volume was immediately fixed with particle-free paraformaldehyde (2% final concentration) for 12 to 18 h at 4°C. Portions of 1.5 to 4 ml were filtered onto white polycarbonate membrane filters (type GTP; pore size, 0.2 µm; diameter, 25 mm; Millipore). The filters were then carefully rinsed with sterile Milli-Q water, air dried, and stored at −80°C until further processing. The total inorganic carbon content of both habitats was determined by a coulometric single-operator multimetabolic analyzer (SOMMA) system (17). For samples from the Black Sea, the mean inorganic carbon concentration accounted for 3,300 µmol/kg; for those from the Baltic Sea, the concentration was 1,950 µmol/kg. Dark CO₂ fixation rates were calculated from the total inorganic carbon concentration, the initial amount of [¹⁴C]bicarbonate added, and the amount of [¹⁴C]CO₂ fixed in biomass during the incubation time.

CARD-FISH. CARD-FISH was carried out according to the protocols of Pernthaler et al. (33) and Sekar et al. (35), modified as described previously (13). For enumeration of Bacteria, a mix of probes EUB338 (GCTGCCCTCCTAGAG) (3), EU3B38-II (GCGAGCACCCTAGTGTG), and EU3B38-III (GCTGCCACCCGTAGGTGT) (8) was used. Epsilonproteobacteria were detected with probe EPS914 (25), and the specific Sulfitobacter subgroup GD17 was detected with probe SUL90 (GCTGCCCGCTAATACTCA) (13). Probe EPS914 (GCTGCCCGCTTATCTCCT) was successfully tested for its specificity as described previously (13). Nonspecific binding was determined using the NonEUB probe (ACTCTACCGGGAGGCACG) (44). Negative control counts with probe NonEUB averaged 0.08 to 0.6% for both the Baltic Sea and the Black Sea. In all samples, implying that cell counts in the same order of magnitude could be considered negligible. For hybridization, 400 µl of hybridization buffer (55% formamide for all probes) and 2 µl of probe working solution (50 pmol µl⁻¹) were mixed. For probe mix EU3B38-I/III, 600 µl of hybridization buffer and 3 µl of probe working solution were used. Hybridization was carried out at 35°C for 8 to 12 h on a rotary shaker in the dark. After the filter sections had been washed in a prewarmed washing buffer (37°C) for at least 10 min, the tyramide signal amplification with 5- and 6-carboxyfluorescein-labeled tyramides was carried out for 15 min in the dark on a rotary shaker. The filters were then washed first in phosphate-buffered saline and afterwards in ethanol and then air dried.

Microautoradiography. The autoradiographic procedure followed the protocol of Teira et al. (41), modified according to Alonso and Pernthaler (1). Hybridized filter sections were glued onto glass slides (UHU Plus Sofortfest; UHU GmbH, Germany), which were then dipped into the photographic emulsion (Kodak; type NTB-2; melted at 43°C for 20 min) and placed in a light-tight box with silica gel as a drying agent. Optimal exposure times at 4°C were 2 days for the Baltic Sea samples and 3 days for the Black Sea samples. The slides were developed (Dektol developer) and fixed (Kodak fixer) according to the specifications of Kodak. The completely dry filter sections were counterstained with the previously described mixture of 4’,6’-diamidino-2-phenylindole (DAPI), Citifluor, and Vectashield (33).

Microscopy. Filter sections were examined with an epifluorescence microscope (Axioskop 2 MOT Plus; Zeiss) equipped with a 100× Plan Achromat objective lens (Zeiss) and appropriate filter sets for DAPI and fluorescein isothiocyanate (FITC). The transmission mode of the microscope allowed the detection of silver grains attached to cells. Cells associated with two or more silver grains were defined as [¹⁴CO₂] positive. In negative control samples, the percentage of cells attached to at least two silver grains accounted, on average, for only 1% of all DAPI-stained cells for the Baltic Sea redoxcline and 0.6% for the Black Sea redoxcline. Switching between fluorescence and transmission modes allowed probe-hybridized [¹⁴CO₂]-positive and inactive cells to be counted directly, followed by the determination of DAPI-stained cells to estimate the total prokaryotic abundance. Between 500 and 1,000 DAPI-stained cells in randomly distributed microscopic fields were counted for each filter section. Bacterial counting of DAPI-stained samples and hybridized samples is usually done with a standard deviation of less than 7.5% for replicates of the central Baltic Sea redoxcline (13).

Results

Bacterial abundance and activity in the Baltic Sea. The chemocline, defined as the shallowest appearance of hydrogen sulfide, was located at a depth of 136 m in the Gotland Deep in April 2007 (Fig. 1). Oxygen was not detected below a depth of 121 m; nitrate was below the detection limit at a depth below 132 m. Dark CO₂ fixation rates below the chemocline ranged from 1.0 to 2.2 µmol of C liter⁻¹ day⁻¹; the highest rate was measured at a depth of 142 m. Prokaryotic abundance, determined by DAPI, remained constant across the three depth layers, at around 1 × 10⁸ cells ml⁻¹ (Fig. 2A). MICRO-CARD-FISH was applied to visualize the [¹⁴CO₂]-fixing activity of individual prokaryotic cells, and [¹⁴CO₂]-positive cells were enumerated for the central Baltic Sea. [¹⁴CO₂]-positive cells, ranging between 1.2 × 10⁶ and 3.1 × 10⁶ cells ml⁻¹, accounted for 12.2 to 29.0% of the total cell abundance (Fig. 2A). Nearly all [¹⁴CO₂]-positive cells were positive for probe EUB338I-III and were consequently assigned to Bacteria. The use of probe EPS914, specific for Epsilonproteobacteria, revealed that [¹⁴CO₂]-positive Epsilonproteobacteria contributed between 63 and 77% of the total number of [¹⁴CO₂]-fixing Bacteria at all depths examined. Hence, in the Gotland Deep, Bacteria taking up [¹⁴CO₂] belonged mainly to Epsilonproteobacteria and, more
specifically, to the *Stoichiimonas* subgroup GD17 (Fig. 2B). Generally, the abundance of GD17 cells with respect to total cell abundance was in the same order of magnitude as for *Epsilonproteobacteria*; thus, members of group GD17 predominated within this group (Table 1). Concerning the proportion of $^{14}$CO$_2$-positive GD17 cells, no more than 65% of total GD17 cells were $^{14}$CO$_2$-fixing at all depths examined (Table 1).

**Bacterial abundance and activity in the Black Sea.** In the Black Sea redoxcline, the chemocline was located at a depth of 110 m (Fig. 3). Nitrate and oxygen were below the detection limit at a depth of more than 90 m. The pronounced maximum amount of dark CO$_2$ fixation rates was detected at a depth of 115 m (Fig. 3). Total prokaryotic abundance ranged between $0.45 \times 10^9$ and $0.72 \times 10^9$ cells ml$^{-1}$; between $0.1 \times 10^9$ and $0.95 \times 10^9$ cells ml$^{-1}$ were identified as $^{14}$CO$_2$ positive, constituting up to 12.8% of total DAPI counts (Fig. 4A). As in the Baltic Sea, MICRO-CARD-FISH of Black Sea samples revealed that $^{14}$CO$_2$-fixing activity was exclusively due to *Bacteria*, i.e., EUB338I-III-positive cells. *Epsilonproteobacteria* taking up $^{14}$CO$_2$ constituted 24 to 100% of all $^{14}$CO$_2$-positive cells, indicating their dominance in dark CO$_2$ fixation among *Bacteria* (Fig. 4B; also see Fig. S1 in the supplemental material). $^{14}$CO$_2$-positive GD17 cells were only detected at a depth of 120 m and amounted to only 1.6% of all DAPI-stained cells. The contribution of group GD17 cells to total cell abundance was low and below the detection limit for deeper layers (Table 1) but accounted for, on average, 12% of total epsilonproteobacterial abundance in the upper layers. $^{14}$CO$_2$-assimilating cells within the

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**TABLE 1. Proportion of Bacteria, Epsilonproteobacteria, and group GD17 cells as percentages of total cell abundance (DAPI counts) in the Baltic Sea and Black Sea redoxclines.$^a$**

<table>
<thead>
<tr>
<th>Location</th>
<th>CARD-FISH</th>
<th>MICRO-CARD-FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depth (m)</td>
<td>EUR (%)</td>
</tr>
<tr>
<td>Baltic Sea</td>
<td>142 70.8  16.9  18.1  62.8  56.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>144 59.9  27.9  31.8  78.2  65.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>156 64.3  21.7  19.7  64.0  62.0</td>
<td></td>
</tr>
<tr>
<td>Black Sea</td>
<td>110 54.1  21.0  1.9  33.0  3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>115 58.5  12.2  1.6  33.6  10.7</td>
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<tr>
<td></td>
<td>120 52.1  35.1  4.9  41.9  32.7</td>
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<tr>
<td></td>
<td>125 54.7  10.9  —   61.0  —</td>
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<tr>
<td></td>
<td>130 56.8  2.9   —   41.9  —</td>
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<td></td>
<td>135 26.6  3.0   —   9.3   —</td>
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$^a$ The percentages of $^{14}$CO$_2$-positive *Epsilonproteobacteria* with respect to total epsilonproteobacterial cell numbers and the percentages of $^{14}$CO$_2$-positive GD17 cells with respect to total GD17 abundance are also given. Results are based, respectively, on CARD-FISH and MICRO-CARD-FISH analyses. EUR, *Bacteria*; EPS, *Epsilonproteobacteria*; —, below detection limit.

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**FIG. 2. Abundances of total and $^{14}$CO$_2$-assimilating prokaryotic cells (DAPI) (A) and percentages of $^{14}$CO$_2$-assimilating *Bacteria* (EUB), *Epsilonproteobacteria* (EPS), and group GD17 cells (GD17) with respect to total cell abundance, determined by MICRO-CARD-FISH (B), in the sulfidic area of the central Baltic Sea in April 2007.**

**FIG. 3. Concentrations of H$_2$S, O$_2$, and NO$_3^-$ and dark CO$_2$ fixation rates in the Black Sea redoxcline in May 2007. The dashed horizontal line indicates the chemocline.**

**FIG. 4. Abundances of total and $^{14}$CO$_2$-assimilating prokaryotic cells (DAPI) (A) and percentages of $^{14}$CO$_2$-assimilating *Bacteria* (EUB), *Epsilonproteobacteria* (EPS), and group GD17 cells (GD17) compared to total cell abundance, determined by MICRO-CARD-FISH (B), in the sulfidic area of the Black Sea in May 2007.**
**Epsilonproteobacteria** ranged from 33.0 to 61.0% of the total *Epsilonproteobacteria* in the zone characterized by the highest dark CO₂ fixation rates (110 to 125 m) (Table 1).

**DISCUSSION**

The results of this study provide direct evidence for the quantitative significance of *Epsilonproteobacteria* in dark CO₂ fixation in sulfidic areas of two marine redoxclines. In the sulfidic area of the Baltic Sea as well as the Black Sea redoxcline, cells taking up ¹⁴CO₂ were identified by MICRO-CARD-FISH as *Bacteria*; more specifically, they were mainly composed of *Epsilonproteobacteria*. In this study, the fraction of ¹⁴CO₂-assimilating *Bacteria* in the Baltic Sea was two times higher than the highest corresponding percentage obtained for the Black Sea. Similarly, dark CO₂ fixation rates for the Baltic Sea were substantially higher than those determined for the Black Sea, albeit comparable to previously reported rates (13, 18, 21, 37). The total abundance of *Epsilonproteobacteria* in the Black Sea and, more specifically of group GD17 cells in the Gotland Deep, was consistent with previous CARD-FISH results, which showed high abundance and a wide depth distribution (13, 24). Likewise, the layer with the highest abundance of CO₂-fixing cells was located in the sulfidic area, with a sulfide concentration of 8 to 10 μmol liter⁻¹ for both habitats. For the Baltic Sea, the number of cells taking up ¹⁴CO₂ generally agreed with the calculations of Jost et al. (19), who estimated between 20 and 40% chemooautrophic cells.

The importance of bacterial chemooautotrophic production, measured as dark CO₂ fixation, has been discussed for different marine redoxclines in relation to phototrophic primary production (34, 39). Combining dark CO₂ fixation measurements with the number of ¹⁴CO₂-positive cells assessed by MICRO-CARD-FISH allows cell-specific dark CO₂ fixation rates to be estimated for chemooautrophic cells, assuming CO₂ as the sole carbon source. The anaplerotic uptake of CO₂ by heterotrophic bacteria was considered to be insignificant for chemooautotrophic CO₂ fixation, as outlined by Jost et al. (19) and Taylor et al. (40). Based on these assumptions, cell-specific dark CO₂ fixation rates would amount to 61 to 217 fg of C cell⁻¹ day⁻¹ for the Baltic Sea redoxcline and 61 to 115 fg of C cell⁻¹ day⁻¹ for the Black Sea redoxcline. These rates are unrealistically high and also not in line with the cell-specific CO₂ uptake rates of 10 to 25 fg of C cell⁻¹ day⁻¹ calculated for a Baltic Sea redoxcline by Jost et al. (19) after flow-cytometric sorting of ¹⁴CO₂-labeled cell clusters. However, those authors based their calculation on a higher abundance of CO₂-fixing cells than was found in the present study using MICRO-CARD-FISH. It is possible that the amount of ¹⁴CO₂-positive cells reported here was underestimated due to the detection limit of microautoradiography and to possible leakage of incorporated radioactivity during fixation and storage (31). Furthermore, protists that were grazing on chemooautotrophic bacteria during incubation or were harboring chemooautotrophic ecto- and endosymbiotic bacteria could have reduced the amount of ¹⁴CO₂-positive cells visible after MICRO-CARD-FISH. It has been reported previously that the standard CARD-FISH procedure is destructive to protist cells (29).

Chemooautotrophic *Archaea* have recently been identified in mesopelagic waters of the North Atlantic (15) and elsewhere (16, 20). Almost all ¹⁴CO₂-positive cells in the dark CO₂ fixation maxima of the Baltic Sea and Black Sea redoxclines belonged to *Bacteria*, precluding a major archaeal contribution to the chemooautotrophic community in the sulfidic areas of these redoxclines. As mentioned above, for the Baltic Sea, Glaubitz et al. (10) investigated the incorporation of ¹³C into chemooautotrophic cells by rRNA-SIP. The authors could not identify autotrophic *Archaea*; however, autotrophic activity of *Gamma-proteobacteria* in addition to that of *Epsilonproteobacteria*, mostly belonging to group GD17, was shown. Consequently, for the Baltic Sea, it is likely that the remaining proportion of unidentified ¹⁴CO₂-fixing cells in the present study consisted, at least partly, of *Gamma-proteobacteria*. However, hybridizations with probe GAM42a (28) did not detect chemooautrophic cells in samples from the central Baltic Sea; however, this may have been due to sequence mismatch (our unpublished results) (2).

Campbell et al. (5) emphasized the potential role of *Epsilonproteobacteria* for biogeochemical cycles, especially at the oxic-anoxic interfaces. For deep-sea hydrothermal fields, chemooautotrophic activity by *Epsilonproteobacteria* has been demonstrated by different authors (30, 32, 38). Lin et al. (23, 24) showed elevated epsiproteobacterial abundances but low epilsonproteobacterial ³H-leucine assimilation activity for the Cariaco Basin and proposed chemooautotrophic activity of *Epsilonproteobacteria* there. *Epsilonproteobacteria* constitute 75 to 100% of ¹⁴CO₂-assimilating *Bacteria* in the layers of highest dark CO₂ fixation in this study, and they apparently contributed substantially to chemooautotrophic production in both the Black Sea and Baltic Sea redoxclines and can be regarded as key organisms for chemooautotrophic production. Therefore, the question arises as to whether or not *Epsilonproteobacteria* are globally important chemooautotrophs in marine habitats where high dark CO₂ fixation rates around redoxclines have been measured.

Among ¹⁴CO₂-positive *Epsilonproteobacteria*, members of the *Sulfurimonas* subgroup GD17 were the dominant representatives in the sulfidic area of the Baltic Sea, in contrast to the rather low contribution of group GD17 to *Epsilonproteobacteria* in the Black Sea. Grote et al. (13) discussed the possible contribution of group GD17 to autotrophic activity, even though quantitative PCR data suggested a restricted high-activity zone of this group around the chemocline several meters above the dark CO₂ fixation maximum. However, the results of this study evidence the major contribution of group GD17 to dark CO₂ fixation in the sulfidic area of the Baltic Sea. Still, the prevailing metabolism for chemooautotrophy in marine sulfidic areas is unknown, and the availability of electron acceptors for chemooautotrophy has been discussed by several authors previously (13, 18, 40). Dark CO₂ fixation driven by autotrophic denitrification with reduced sulfur compounds as electron donors is unlikely, since there was no evidence for a clear overlap between nitrate and hydrogen sulfide. The oxidation of reduced sulfur species combined with the reduction of particulate metal oxides might be possible in the Baltic Sea and in the Black Sea (18, 19). Members of group GD17 were initially regarded as sulfur-oxidizing denitrifiers, but the remaining high cell numbers in sulfidic nitrate-deprived waters suggested a possible metabolic versatility for this group (4, 13). Notably, the percentage of ¹⁴CO₂-assimilating epsilon-
proteobacterial cells in layers with the highest dark CO₂ fixation rates never exceeded 65% of total Epsilonproteobacteria, leaving 35% of the cells either metabolically inactive or heterotrophic. As mentioned above, Lin et al. (23) detected a significant number of heterotrophic Epsilonproteobacteria in the Cariaco Basin. These findings stress the potential for heterotrophic as well as autotrophic activity within the Epsilonproteobacteria at marine redoxlines. Moreover, considering the Baltic Sea redoxcline, with group GD17 as the dominant epsilonproteobacterial representative, it is likely that group GD17 is metabolically versatile, exhibiting chemautotrophy and, potentially, heterotrophy. The ability of members of GD17 to switch between heterotrophic and autotrophic metabolism could also explain its wide depth distribution, from the suboxic to the sulfidic layers, as reported by Grote et al. (2003). Use of combined microautoradiography and fluorescence in situ hybridization to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus *Achromatium*. Appl. Environ. Microbiol. 66:4518–4522.


