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Dominating Role of an Unusual Magnetotactic Bacterium in the Microaerobic Zone of a Freshwater Sediment

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A combination of polymerase chain reaction-assisted rRNA sequence retrieval and fluorescent oligonucleotide probing was used to identify in situ a hitherto unculturable, big, magnetotactic, rod-shaped organism in freshwater sediment samples collected from Lake Chiemsee. Tentatively named "*Magnetobacterium bavaricum*," this bacterium is evolutionarily distant from all other phylogenetically characterized magnetotactic bacteria and contains unusually high numbers of magnetosomes (up to 1,000 magnetosomes per cell). The spatial distribution in the sediment was studied, and up to 7×10^5 active cells per cm^3 were found in the microaerobic zone. Considering its average volume (25.8 ± 4.1 μm^3) and relative abundance ($0.64 \pm 0.17\%$), "*M. bavaricum*" may account for approximately 30% of the microbial biovolume and may therefore be a dominant fraction of the microbial community in this layer. Its microhabitat and its high content of sulfur globules and magnetosomes suggest that this organism has an iron-dependent way of energy conservation which depends on balanced gradients of oxygen and sulfide.

Magnetotactic bacteria have the ability to orient themselves along the lines of magnetic fields. Since these organisms were discovered by Richard Blakemore in the mid-1970s, many other researchers have found them in aquatic environments and wet soils (for a review, see reference 18). The production of magnetosomes by these bacteria may contribute significantly to the natural remanent magnetism of sediments (16, 23). A variety of morphologically and metabolically diverse magnetotactic bacteria (reviewed in reference 4) are easily enriched by taking advantage of their magnetic properties (19). However, only a very few isolates exist in axenic culture, and consequently only two of these bacteria have been phylogenetically characterized by 16S rRNA sequence analysis (10, 29). Unfortunately, the previously validly described, culturable organisms *Magnetospirillum magnetotacticum* (formerly *Aquaspirillum magnetotacticum*) and *Magnetospirillum gryphiswaldense*, both small spirilla with only a few magnetosomes, are rarely seen in environmental samples. Other morphotypes (e.g., cocci, bacilli, and vibrios of various sizes having various magnetosome contents) are encountered much more frequently.

Several years ago, an unusual, large, magnetotactic, rod-shaped organism containing extremely high numbers of magnetosomes was described phenotypically (35). So far, this organism has been enriched only from the calcareous sediments of several freshwater lakes in Upper Bavaria, Germany, and it has been tentatively named "*Magnetobacterium bavaricum*." As is the case for many other magnetotactic bacteria, microbiologists were unable to grow this bacterium in pure culture until now. This is probably because many of the magnetotactic bacteria are typical gradient organisms and require, for example, microaerobic conditions for optimal growth. Recently developed methods for in situ characterization of bacteria without prior cultivation (1, 31) were used to study the phylogeny and spatial distribution of this unusual magnetotactic bacterium.

MATERIALS AND METHODS

Sampling, enrichment, and cell sorting. Samples were collected from Lake Chiemsee at a depth of 15 to 20 m. This big freshwater lake in Upper Bavaria, Germany, has a rather high pH (pH 8.3) and 100% oxygen saturation in the water column. Samples including the upper few centimeters of the calcareous sediment were transferred to aquaria and stored at room temperature protected from direct light. After several weeks, samples were taken right beneath the water-sediment interface and used for enrichments by imposing a magnetic field with a bar magnet (31). We either collected a suspension of various magnetotactic bacteria or immobilized cells on glass slides for in situ hybridization. Forward and large-angle scatter analyses allowed us to sort the magnetic cell suspensions obtained with a FACS Star Plus flow cytometer (Becton Dickinson, Mountain View, Calif.) into two discrete subpopulations.

Polymerase chain reaction amplification, cloning, and sequencing. Sorted cells were directly used for in vitro amplification (26, 28) of 16S rRNA genes. We amplified nearly complete 16S rRNA gene sequences with a generic primer set (5′-AGAGTTTGATYMTGGCTCAG-3′, corresponding to *Escherichia coli* 16S rRNA positions 8 to 27; 5′-CAKAA AGGAGGTGATCC-3′, corresponding to *E. coli* 16S rRNA positions 1528 to 1544). The amplified products were singularized by cloning them in plasmid vector pBluescript (Stratagene, La Jolla, Calif.), and subsequently the 16S rRNA sequences were determined by using the chain termination technique (6).

Cell fixation and in situ hybridization. Cells immobilized in situ on microscope slides were subsequently fixed in an ethanol series (50, 80, and 98% ethanol for 3 min each). The slides were air dried and stored at room temperature.

rRNA-targeted fluorescent oligonucleotide probes permit

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in situ identification of individual microbial cells (3, 8). Probes were synthesized and purified as described previously (3). They were diluted in hybridization solution (0.9 M sodium chloride, 0.01% sodium dodecyl sulfate, 20 mM Tris hydrochloride; pH 7.2) to final concentrations of 2.5 ng/μl for fluorescein-labeled probes and 1 ng/μl for tetramethylrhodamine-labeled probes. Aliquots (20 μl) were placed on the slides and covered with glass coverslips, and the preparations were incubated at 46°C for 75 min in an isotonically equilibrated humid chamber. The coverslip and probe were removed with hybridization solution, and the slide was incubated in 40 ml of hybridization solution (without probe) for 20 min at 48°C. The slides were rinsed with distilled water, air dried, and mounted in Citifluor solution (Citifluor, London, United Kingdom).

Determining cell counts. Sediment cores were divided into 3-mm increments. Total cell counts were determined by using the 4′,6-diamidino-2′-phenylindole (DAPI; Boehringer, Mannheim, Germany) method (24). Sediment samples were stained by using a final DAPI concentration of 0.33 μg/ml for 30 min at room temperature and were subsequently transferred to polycarbonate filters (pore diameter, 0.2 μm; Reichelt, Heidelberg, Germany) which had been counterstained with Irgalan Black (Ciba Geigy, Wehr, Germany). Cells of "*M. bavaricum*" were counted after magnetic separation and in situ hybridization.

Epifluorescence microscopy. Cells were viewed with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence with a 50-W mercury high-pressure bulb and filter sets 01, 09, and 15. Photomicrographs were made with Kodak Tmax 400 film. The exposure times were 0.04 s for phase-contrast micrographs and 15 to 30 s for epifluorescence micrographs.

Electron microscopy. The bacteria from the enrichments were fixed for electron microscopy as described previously (29).

Sequence alignment and phylogenetic tree. The new sequence which we determined was added to an alignment of more than 800 bacterial 16S rRNA sequences. The sequence data were obtained from the available rRNA sequence data bases (9, 21). Similarity and distance matrices were calculated from this data set by using the SEQDIS program (33a). Phylogenetic distances were calculated as described by Jukes and Cantor (15). Distance matrix trees were constructed by using the neighbor-joining method of Saitou and Nei (27) and the NEIGHBOR program as implemented in Felsenstein's PHYLIP package (12). Parsimony analyses of the complete data set were performed by using Felsenstein's DNAPARS program. Various data sets that differed with respect to the alignment positions included or the selection of sequences were analyzed. Alignment positions were selected according to their degrees of sequence conservation as determined by the SEQDIS program. Maximum-likelihood analyses were performed with subsets of the data by using Olsen's fastDNAml program (21). Branchings for which a relative order could not unambiguously be determined by using different treeing methods and different data subsets are shown as multifurcations.

Measurements of oxygen and sulfide with needle electrodes. Needle electrodes were constructed and calibrated as described previously (36, 37). The oxygen microelectrode was connected to a calomel reference electrode and a picoammeter with a 750-mV polarization charge positive to the calomel electrode, whereas the sulfide microelectrode required a millivoltmeter with high impedance (10^{15} Ω). Both meters were custom made by the Electronics Workshop at the University of Groningen. The microelectrodes were mounted on a motor-driven micromanipulator (model MM33; Märzhäuser, Wetzlar, Germany) which was placed on a sturdy aluminum stand. Profiles were obtained in downward direction in 100-μm increments starting several millimeters above the sediment and continuing to a depth of 15 mm.

RESULTS AND DISCUSSION

Detailed phenotypic description. The tentative name "*M. bavaricum*" was given to magnetotactic rod-shaped organisms that were 8 to 10 μm long and 1.5 to 2 μm in diameter and contained exceptionally high numbers of magnetosomes (up to 1,000 magnetosomes per cell) (Fig. 1). This is far more magnetosomes than would be required for orientation along the lines of the earth's magnetic field. The magnetosomes are projectile shaped (length, 110 to 150 nm) and have an average magnetic moment of 85×10^{-18} Am^2, assuming that the magnetosomes consist of magnetite (saturation magnetization, 48×10^4 A m^{-1}). The magnetosomes are arranged in several straight chains that are parallel to the long axis of the bacterium, and the average magnetic moment is 32×10^{-15} Am^2 per cell. The large cells are gram negative and often contain other inclusions, which have been demonstrated to be sulfur by their solubility in methanol (Fig. 1) (33) and energy-dispersive X-ray analysis. The cells of "*M. bavaricum*" have one polar tuft of flagella that can be visualized in living cells by phase-contrast microscopy. When a cell is swimming forward (toward the North Pole), the flagella are wound around the rotating cell; when a cell is swimming in the opposite direction, the flagellar tuft is reversed and propels the cell. The average speed of cells swimming forward is 40 μm/s, a value that is considerably higher than the speed in the opposite direction. Under the influence of an imposed magnetic field "*M. bavaricum*" responds to chemical gradients by reversing its swimming direction for short periods of time.

In situ identification and phylogeny of "*M. bavaricum*." Previous attempts to determine the 16S rRNA sequence of "*M. bavaricum*" by using mixed magnetic enrichment cultures failed. Although microscopic examination revealed high numbers of "*M. bavaricum*" cells in the enrichment cultures, all of the sequences obtained after a polymerase chain reaction and cloning could be assigned to magnetic cocci or contaminating nonmagnetic bacteria (31). By taking advantage of the characteristic size and magnetosome content, big cells could be sorted from smaller cocci and vibrios with a flow cytometer. This fraction was directly used for polymerase chain reaction amplification and cloning. A new 16S rRNA sequence was found in seven of nine clones. A fluorescent oligonucleotide complementary to a diagnostic region of this sequence hybridized only to cells of "*M. bavaricum*" (Fig. 1d, e, and f). Unlike the magnetic cocci, whose phenotypically identical cells exhibited genotypic heterogeneity, the morphotype "*M. bavaricum*" consisted of only one population with one genotype. All of the sequences which we determined were identical, and all of the cells hybridized to the probe that targeted a variable region. The 16S rRNA sequence of "*M. bavaricum*" has been deposited in the EMBL data base under accession number X71838. A comparative 16S rRNA sequence analysis showed that there was only a distant relationship between "*M. bavaricum*" and all other magnetotactic bacteria for which 16S rRNA sequences have been determined. The corresponding overall levels of sequence similarity are

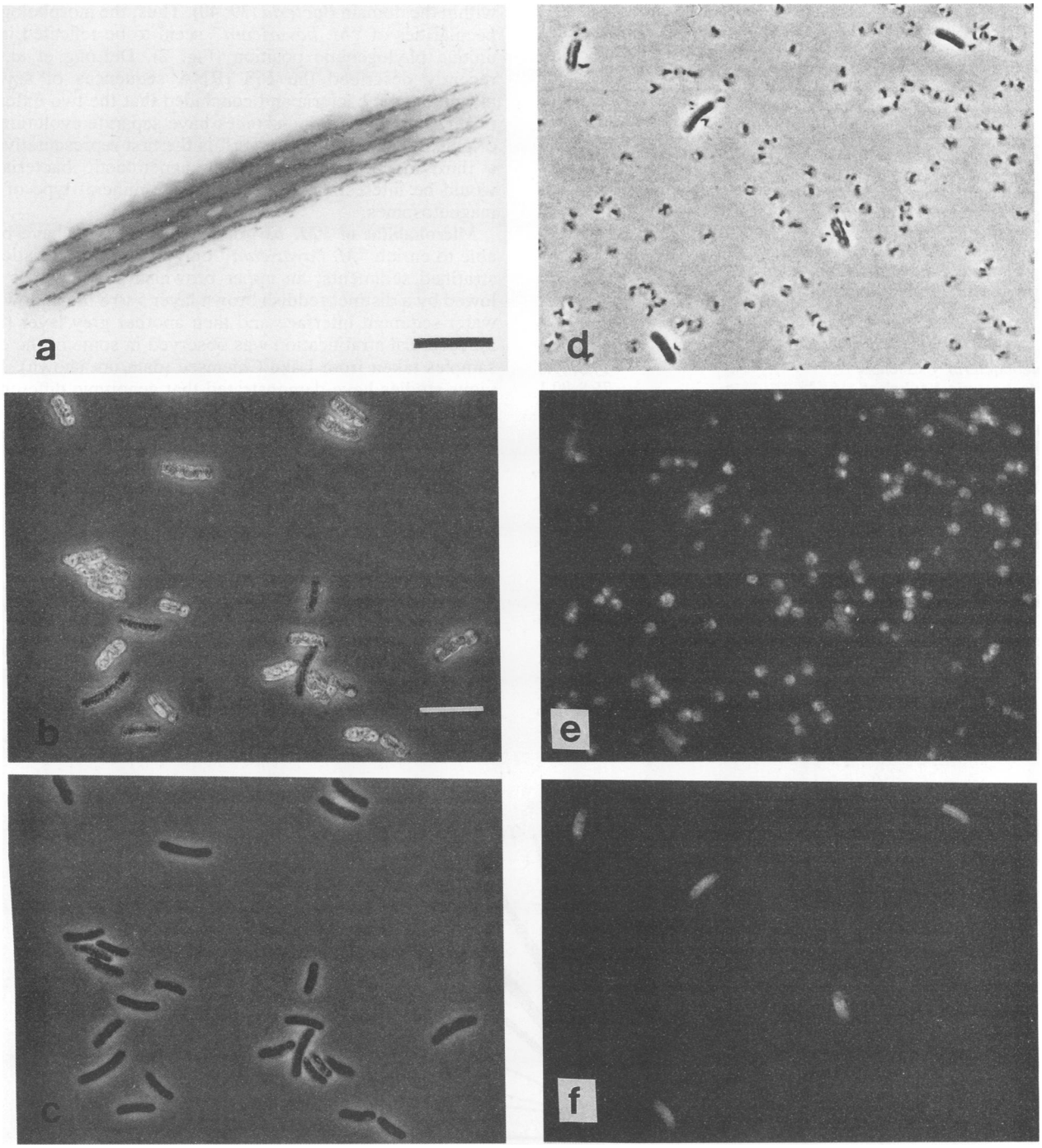

FIG. 1. (a) Electron micrograph of "*M. bavaricum*" cell with multiple chains of magnetosomes. Bar = 1 μm. (b and c) Identical microscopic fields. Bar = 10 μm. Phase-contrast micrographs reveal sulfur globules in "*M. bavaricum*" (b) which can be completely removed with methanol (c). (d through f) Whole-cell hybridization of "*M. bavaricum*": fixed magnetic enrichment viewed by phase-contrast microscopy (d) and epifluorescence microscopy in which fluorescein-specific filters (e) and rhodamine-specific filters (f) were used. All cells bound a fluorescein-labelled bacterial probe (2); only "*M. bavaricum*" cells bound the tetramethylrhodamine-labelled specific probe (5′-GCCATCCCCTCGCTTACT-3′).

shown in Table 1 and are in the same range as the levels of sequence similarity found for the "*M. bavaricum*" sequence and all available sequences from representatives of the major lines of descent in the domain *Bacteria*. No convincing signatures could be found to assign the organism to the *Proteobacteria*. In fact, no remarkable sequence similarity could be found to any bacterial 16S rRNA in the available data bases. As Fig. 2 shows, "*M. bavaricum*" is currently either the deepest branching organism of the proteobacterial (32) phylum or may even represent an independent phylum

TABLE 1. Overall levels of sequence similarity between 16S rRNA from "*M. bavaricum*" and 16S rRNAs from other magnetotactic bacteria and the major phylogenetic groups of the domain *Bacteria*[a]

Organism or group	Strain	% Similarity
Magnetospirillum magnetotacticum	DSM 3856^T	78.3
Magnetospirillum gryphiswaldense	DSM 6361^T	79.9
Magnetotactic *Proteobacterium* sp.	CS 103	79.1
Magnetotactic *Proteobacterium* sp.	CS 308	79.6
Magnetotactic *Proteobacterium* sp.	CS 310	78.6
Magnetotactic *Proteobacterium* sp.	MC 1	76.9
Magnetotactic *Proteobacterium* sp.	MV 1	76.5
Magnetotactic *Proteobacterium* sp.	MMP 1990	74.0
Magnetotactic *Proteobacterium* sp.	MMP 1991	76.8
Proteobacteria α subclass		73.2–79.9
Proteobacteria β subclass		73.7–77.0
Proteobacteria γ subclass		74.3–79.0
Proteobacteria δ subclass		75.8–80.1
Proteobacteria ε subclass		73.1–76.0
Gram-positive bacteria with low DNA G+C contents		72.7–80.4
Gram-positive bacteria with high DNA G+C contents		74.0–79.2
Cyanobacteria		74.4–77.1
Spirochetes		73.7–78.2
Planctomycetes		72.7–75.9
Cytophaga-Flavobacterium-Bacteroides group		72.1–76.0
Green sulfur bacteria		78.1–79.4
Deinococci		73.1–79.9
Green nonsulfur bacteria		73.6–74.4
Thermotogales		75.0–77.8

[a] Data for other magnetotactic bacteria were obtained from references 7, 29, and 31. Data for other major groups of *Bacteria* were obtained from reference 21. Ranges of similarity values are given for the phylogenetic groups.

within the domain *Bacteria* (39, 40). Thus, the morphological pecularities of "*M. bavaricum*" seem to be reflected in its unique phylogenetic position (Fig. 2). DeLong et al. (7) recently described the 16S rRNA sequences of several magnetotactic bacteria and concluded that the two different mineral types of magnetosomes have separate evolutionary origins. Since "*M. bavaricum*" is the first representative of a third independent group of magnetotactic bacteria, it would be interesting to determine the mineral type of the magnetosomes.

Microhabitat of "*M. bavaricum*." So far, we have been able to enrich "*M. bavaricum*" only from characteristically stratified sediments; an upper brownish grey layer is followed by a distinct reddish brown layer 5 to 8 mm below the water-sediment interface and then another grey layer (Fig. 3). Identical stratification was observed in some of the core samples taken from Lake Chiemsee (data not shown). Previous studies have demonstrated that genotypic differences among morphologically identical magnetotactic bacteria are reflected in their tactic behavior and spatial distribution in gradients (31). In this study, the fluorescent oligonucleotide probe specific for "*M. bavaricum*" was used for in situ identification. Whereas no cells of "*M. bavaricum*" were detected in the water and the first few millimeters of the sediment, up to 7×10^5 cells per cm^3 were present in the reddish brown layer of the sediment (Fig. 3). The high amount of probe-conferred fluorescence indicated that there was a high rRNA content and consequently high physiological activity (8) of "*M. bavaricum*" in the habitat. Measurements with an oxygen microelectrode revealed that this layer coincided with the microaerobic zone (Fig. 3). Since magnetic separation preceded the enumeration, we probably underestimated the total number of "*M. bavaricum*" cells by counting only the actively swimming population mem-

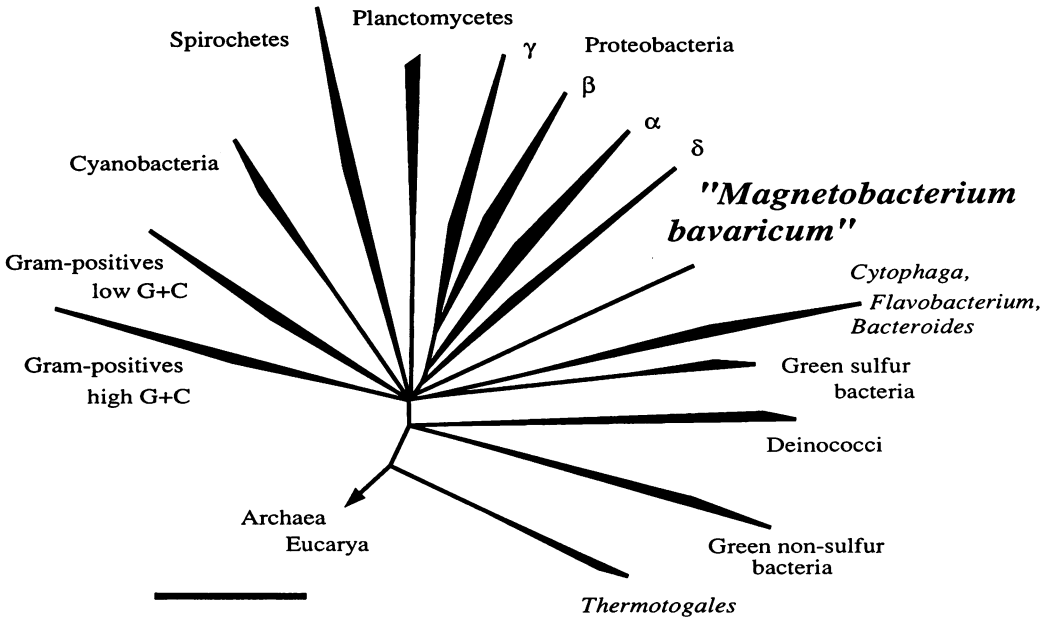

FIG. 2. 16S rRNA-based tree reflecting the phylogenetic relationships of "*M. bavaricum*." The tree is based on the results of a distance matrix analysis of more than 800 bacterial sequences. Since no close relationships between "*M. bavaricum*" and other bacteria could be found, only more conserved alignment positions (i.e., those which were invariant in at least 50% of the available bacterial sequences) were included. The tree was corrected by using the results of parsimony and maximum-likelihood analyses. The multifurcations were drawn to indicate that the relative branching order cannot be unambiguously determined on the basis of presently available data and methods. The solid triangles indicate the phylogenetic depths of major groups of *Bacteria*. The bar represents a phylogenetic distance of 0.05.

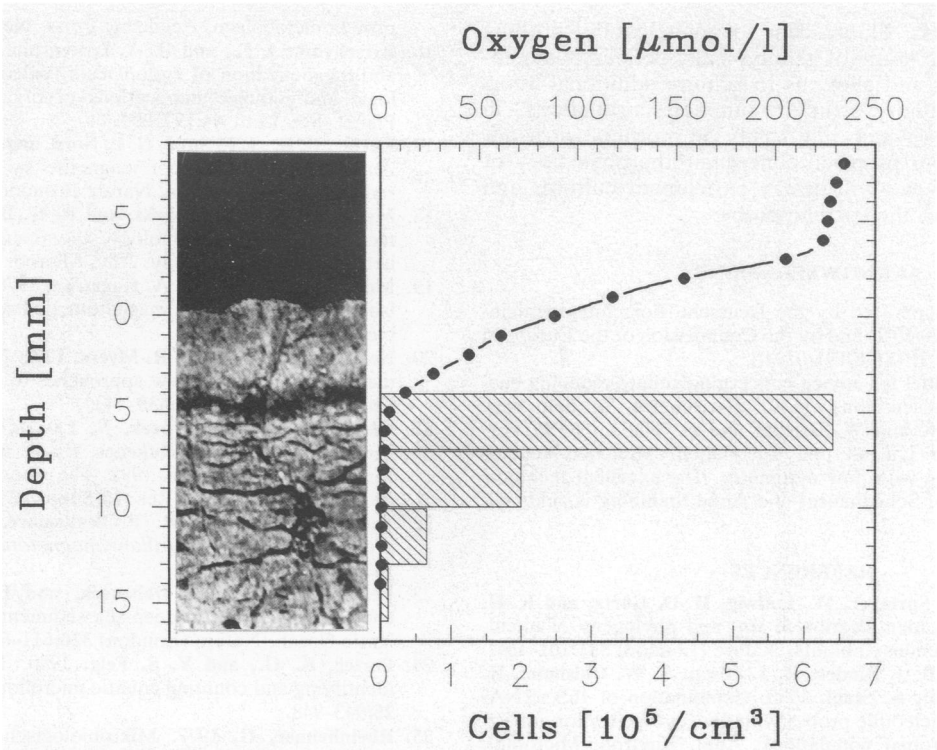

FIG. 3. Vertical distribution of "*M. bavaricum*" in stratified Lake Chiemsee sediment. The cross-hatched bars indicate counts for successive depth fractions (3-mm intervals). Oxygen was measured every 1 mm (solid circles). A photograph of the corresponding section of the aquarium is shown on the left. The water-sediment interface corresponds to 0 mm.

bers. The total cell numbers in the sediment were determined by the DAPI method and were found to be $1.1 \times 10^8 \pm 0.41 \times 10^8$ cells per cm^3. Considering the relative abundance ($0.64 \pm 0.17\%$) and average volume (25.8 ± 4.1 μm^3) of "*M. bavaricum*" cells, which is approximately 50 times greater than the volume of an average microbial cell in freshwater (25), a significant part (approximately 30%) of the microbial biovolume could be attributed to "*M. bavaricum*." Therefore, we suggest that this organism plays a dominating role in the microbial ecology of this sediment layer.

Using sulfide electrodes, we could not detect free sulfide in the sediment. The detection limit of our electrodes was around 10 μM (14). However, enrichment studies (38) have proven that sulfate-reducing bacteria are present in the microaerobic zone. Most likely, sulfate reduction continuously produces hydrogen sulfide, but the concentrations are kept low (<1 μM) by biotic or abiotic processes (30).

Implications for the physiology of "*M. bavaricum*." Our knowledge of the microhabitat and the inclusions allows us to speculate on the physiology of the magnetotactic bacterium "*M. bavaricum*." Recently, there have been indications that oxidation of sulfide occurs in anaerobic nitrate-free sediments (11). The mechanism has not been elucidated yet. However, it has been suggested that there are chemolithotrophic bacteria that use reduced sulfur species for reduction of iron or manganese oxide (34). Enrichment of such organisms is complicated by the rapid chemical reaction between hydrogen sulfide and iron oxides. Not surprisingly, no such bacteria have been identified yet. The high intracellular contents of sulfur and iron make it plausible that "*M. bavaricum*" could be a representative of this important group of gradient microorganisms. The components required for this new type of metabolism (iron oxides, oxygen, and hydrogen sulfide) are all present in the suboxic zone. Lovley et al. (17) have shown that "*Geobacter metallireducens*" (formerly strain GS-15) derives energy from the anaerobic oxidation of organic compounds with Fe(III) as the terminal electron acceptor, resulting in the production of extracellular magnetite. Also, a facultatively chemolithotrophic, hydrogen-oxidizing, Fe(III)-reducing microorganism has already been described (5). These processes have been recently reviewed (20). For "*M. bavaricum*" we propose an alternate pathway in which hydrogen sulfide is used as a reductant coupled to intracellular redox cycling of iron. Indeed, it has recently been shown that redox cycling of iron is a possible mechanism of energy conservation in magnetotactic bacteria (13). In "*M. bavaricum*" the reduction of Fe(III) to Fe(II) during transfer over the cytoplasmic membrane (22) could be coupled to the oxidation of sulfide through an electron transport chain. As in many chemolithotrophic sulfur oxidizers (e.g., *Beggiatoa* spp.), sulfur would be deposited intracellularily, and this sulfur would serve as a reservoir for further reductions. A second electron transport chain could be located in the magnetosome membrane. Here, cytoplasmatic Fe(II) is oxidized to Fe(III) and simultaneously precipitated as amorphous hydrated ferric oxide, which is probably chemically transformed to magnetite. The electrons could be transferred to oxygen, possibly providing energy as in other iron-oxidizing bacteria. Both electron transport chains are thermodynamically possible and could via a proton gradient result in the formation of ATP.

Future directions. Using molecular techniques, we have conclusively demonstrated that magnetotactic bacteria are certainly not an evolutionary curiosity but may play a major role in the microbial ecology of the microaerobic zones of

aquatic sediments. Fluorescent oligonucleotide probing could facilitate the study of genotypically defined magnetotactic populations and allow us to acquire additional information on community structure-function correlations, in contrast to studies which rely solely on morphological differences. Using our proposal concerning the physiology of "*M. bavaricum*," we will design enrichment cultures and evaluate them with the specific probe.

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