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Application of Antisera Raised against Sulfate-Reducing Bacteria for Indirect Immunofluorescent Detection of Immunoreactive Bacteria in Sediment from the German Baltic Sea

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Polyclonal rabbit antisera raised against sulfate-reducing bacteria (SRB) could detect several distinct populations of bacteria in sediment from the German Baltic Sea. The depth distribution of immunoreactive bacteria was determined by an indirect immunofluorescence filter method. Anti-*Desulfovibrio desulfuricans* DSM 1926 serum showed maximum bacterial numbers at a depth of 18 cm, with a concentration of 60×10^6 cells cm^{-3}. With anti-*Desulfovibrio baculatus* DSM 2555 serum, counts were highest at the same depth, approaching 0.7×10^6 cells cm^{-3}. Other significantly smaller populations were observed. Anti-$SRB_{Strain\ 1}$ (lactate,vibrio) maxima were at 0 to 4 cm and at 17 to 18 cm. Anti-$SRB_{Strain\ 2}$(lactate,vibrio) serum showed several local maxima. Anti-$SRB_{Strain\ 3}$(lactate,oval) serum detected one single peak at a depth of 10 to 12 cm. Also determined were rates of sulfate reduction, total bacterial counts by acridine orange staining, and the viable counts by dilution series on anaerobic lactate medium. The total bacterial counts were highest (180×10^6 cells cm^{-3}) at 3 to 4 cm and dropped to 24×10^6 cells cm^{-3} at 10 to 11 cm but showed additional local maxima reaching 140×10^6 cells cm^{-3} at a depth of 17 to 18 cm. Viable counts (most probable number) were above 10^5 CFU cm^{-3} at 0 to 3.6 cm but remained below 10^3 CFU at 7.2 to 18 cm. The sulfate reduction rate was maximal (107 nmol cm^{-3} day^{-1}) at a depth of 1 to 2 cm, dropped to 10 nmol cm^{-3} day^{-1} at 12 to 13 cm, and reached 38 nmol cm^{-3} day^{-1} at 17 to 18 cm.

Dissimilatory sulfate reduction is one of the dominant pathways for the final mineralization of organic carbon in marine sediments (3, 4, 9, 11, 12, 14, 19, 22). The process of sulfate reduction can be monitored directly in the sediment by radiotracer techniques (7, 10, 13), although the rate at which the process occurs in the oxidized zone is underestimated because of rapid H_2S reoxidation by metal ions (iron and manganese) or molecular oxygen. The reactions of hydrogen sulfide, i.e., entrapment by metal ions forming FeS and pyrite (19), or reoxidation to oxidized sulfur compounds such as thiosulfate, elemental sulfur, or sulfate (15) can be chemically or biologically mediated, and in a number of cases a close interrelationship between the sulfide-producing sulfate reducers and the sulfide-consuming sulfide oxidizers (phototropic sulfur bacteria and colorless sulfur bacteria) has been documented (29).

The diversity of described species of sulfate-reducing bacteria (SRB) is rapidly increasing (30), and the extent of their metabolic potential has been realized only recently. Despite the bulk of evidence for the sulfate reduction process in situ (11) and the detailed study of the biochemistry and microbiology of SRB in pure cultures (30), the information on the presence and distribution of the bacteria in marine sediments is less conclusive. Dilution series on different substrates have been used to estimate the numbers and types of SRB (for example, see references 15 and 29). Also, biomarkers such as fatty acids have been used to verify the presence of SRB (21, 25), while 16S rRNA gene (rDNA) sequences amplified with 16S rDNA-selective PCR primers specific for SRB have revealed unknown sequences phylogenetically grouping within the SRB (5). In general, methods for the direct detection and identification of strains of bacteria in marine sediments are urgently needed (18).

The present study describes an immunofluorescent technique using polyclonal rabbit antibodies combined with fluorescein-labelled secondary antibodies to detect SRB in marine sediments. The distribution of immunoreactive bacteria in a silty coastal sediment in the German Baltic Sea is described, and the results are compared with data on the total bacterial number, the number of cultivable SRB (most probable number [MPN]), and the observed sulfate reduction rates.

MATERIALS AND METHODS

Samples. Undisturbed sediment samples were obtained in 26-mm Plexiglas subcores from a box corer cast in August 1994. Samples were collected at position 54°29′26″N, 09°59′15″E at the mouth of Eckernförde Bucht in the German Baltic Sea. The station is characterized by the active deposition of silty material. The depth was 28 m, and the bottom water temperature was 9 to 10°C. The water overlying the sediment was siphoned off, except for a few millimeters, thus exposing the sediment surface to aerated conditions during transport and storage (15). The cores were kept at 4°C until further analyzed in the laboratory during the following 2 weeks.

Microscopy and microphotography. A Zeiss Axioskop microscope, prepared for epifluorescence and equipped with a 50-W Hg lamp plus the filter combinations recommended by Zeiss for fluorescein (excitation, BP 450/490; emission, BP 515/565) and acridine orange (BP 365/12, LP 397) epifluorescence, was used. For stereo microscopy a Zeiss Stemi 2000 microscope was employed.

[^{35}S]sulfate reduction. The sulfate reduction rate was determined by radiotracer techniques as described elsewhere (7). For rate measurement, a Plexiglas subcore with silicone-sealed holes was injected with radioactive sulfate and incubated at 15 to 17°C for 5 h before the core was sliced and the slices were fixed in cold 20% $ZnSO_4$. Reduced sulfur was distilled from the fixed material by boiling with HCl-Cr^{2+} and trapped as ZnS. Sulfate reduction rates were calculated as described elsewhere (13).

Cultivation of bacteria. SRB were grown as described elsewhere (30) in a synthetic bicarbonate-buffered, sulfide-reduced marine medium. The electron donor was in most cases 20 mM lactate. Bacteria were stored at 4°C or as frozen stocks at −80°C. Bacteria were obtained from the DSM (German Collection of Microorganisms and Cell Culture, Braunschweig) or isolated from marine sediments.

Strains. Important for the quantitative technique were the following cultures.

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$SRB_{Strain\ 1}$(lactate,vibrio), a strain cloned from a 10^{-6} dilution tube from an MPN series on lactate, developed at 18°C (North Sea sediment). The strain was rapidly proliferating at this temperature (doubling time, 6 to 7 h) and, according to morphological and physiological criteria, was tentatively classified as a typical *Desulfovibrio* strain. $SRB_{Strain\ 2}$(lactate,vibrio) and $SRB_{Strain\ 3}$(lactate,oval) were recent isolates from the Baltic Sea made by enrichments on lactate medium at 18°C. Each presented only a single morphology by microscopy. $SRB_{Strain\ 2}$(lactate,vibrio) was vibrioid, while $SRB_{Strain\ 3}$(lactate,oval) was a small oval.

Preparation of antisera. Bacterial cultures in mid-log phase were harvested by centrifugation and fixed in 4% formaldehyde in phosphate-buffered saline. Immunization was by twice-weekly subcutaneous injection. Young rabbits were injected with a dense fixed whole-cell bacterial slurry (in buffer without adjuvants). Sera were harvested after 1 to 4 months. Titers and cross-reaction patterns were determined by the microscope slide method described below. The antisera used for quantitative counts were tested for cross-reactivity against (i) deposited strains of SRB, (ii) a wide phylogenetic range of non-SRB, and (iii) a set of bacteria of marine sediment or marine origin (see Table 1).

Bacterial extraction. The bacterial fraction was extracted from sediment slices by suspending a sediment slice (1 cm thick, 26 mm in diameter) in 1 liter of sulfide-reduced medium containing lactate and acetate at 20 mM each. The slurry was homogenized by stirring with a magnetic stirrer for 15 min. The flask was then allowed to stand for 45 min for sedimentation of particulate material. [The extraction efficiency was evaluated by adding $SRB_{Strain\ 1}$(lactate,vibrio) to the sediment, 60% of added bacteria were recovered after immunostaining.] A subsample was taken from the supernatant and fixed by addition of 20% formaldehyde to a final concentration of 2%. The fixed samples were stored at 4°C until further analyzed within the following week.

Viable counts. The enumeration series incubations were done at 15 to 17°C in 6-ml tubes with rubber- or Teflon-sealed screw caps. Subsamples, each covering a 3.6-cm interval at depths between 0 and 18 cm, were taken by using cut-off 1-ml syringes and transferred to sulfide (approximately 1 mM Na_2S)-reduced marine medium containing 20 mM lactate (30), resulting in an initial 10-fold dilution. At each step in the 10-fold dilution series, the sediment was homogenized by repeated passages through 1.5-mm-diameter needles. In contrast to the procedure with the other counting methods, no time was allowed for sedimentation of particles. For each 10-fold dilution, five replicas were used. Bacterial growth was registered by phase-contrast microscopy and sulfate reduction (H_2S production) by the $CuSO_4$-HCl rapid test (30). Numbers were calculated according to the MPN tables in reference 2.

Epifluorescence. Fixed bacterial samples were appropriately diluted in phosphate-buffered saline solution (50 mM sodium phosphate buffer [pH 7.2], 150 mM NaCl) before either addition of acridine orange to the samples for total bacterial determinations (8) or filtering of the samples directly onto black polycarbonate filters for immunostaining. Two versions relying on the same basic principles were developed for the immunofluorescent staining of the bacteria.

The first version used cover slides. To obtain qualitative information, bacteria were adsorbed to gelatin-coated microscopic slides by placing droplets of bacterial suspensions on a slide and allowing some hours for drying. The specific primary antiserum was then added in a droplet covering the bacterial spot. A secondary fluorochrome (fluorescein)-labelled antibody was added after washing of the slide in phosphate-buffered saline. After a final wash, the slide could be observed by epifluorescence microscopy.

The second version used filters. For quantitative studies, bacterial samples were filtered onto black polycarbonate filters (pore width, 0.2 μm; diameter, 25 mm; GTBP 02500; Millipore). Care was taken not to let filters be sucked dry, and the funnel was sealed with Parafilm on top during antibody reactions. The filter, while still in the filter holder, was incubated with the specific primary antiserum, washed by adding buffer to the filter funnel, and then reacted with the secondary fluorescein-labelled antibody. The filter was washed, put on a microscope slide, and finally covered by a coverslip before fluorescent bacteria were counted at a magnification of ×400.

For both immunostaining procedures, the slides were mounted in 50% glycerol–phosphate-buffered saline. Secondary fluorescein-labelled antibodies (swine anti-rabbit) were purchased from Dakopatts, Hamburg, Germany. Antisera were diluted in phosphate-buffered saline–1 mg of bovine serum albumin per ml–5% nonspecific swine serum. All reactions were for 30 to 60 min at 30°C. A subjective intensity scale (maximum, +4) was used to assess the strength of the immunofluorescent reaction (20, 27). In total, 27 antisera were used for screening the sediment.

RESULTS

Sediment. The sediment consisted of silty mud. It was rather homogeneous throughout the entire depth. The surface was yellow-brownish. A brownish zone extended to ca. 1 cm. A black band with macroscopically visible *Beggiatoa* spp. was observed from 1 to about 3 cm. The underlying sediment was greyish. At 10 to 12 cm, another black band was observed but there were no filamentous bacteria. At 16 to 18 cm, a blackening of the sediment, mainly due to small black inclusions

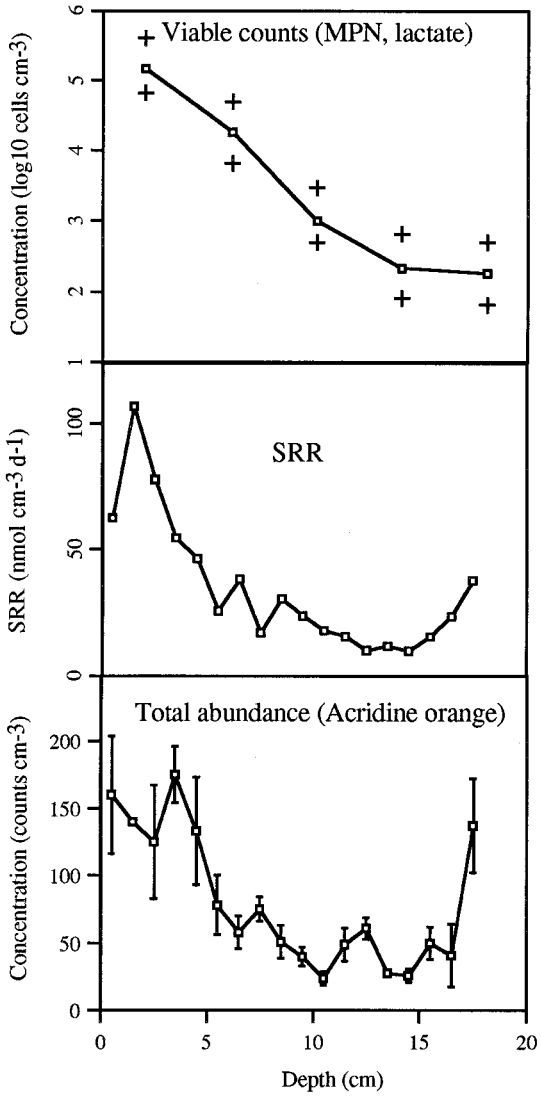

FIG. 1. Depth distribution of viable counts, sulfate reduction rates, and total bacterial numbers. Viable counts (MPN) were determined with anaerobic marine liquid medium supplemented with 20 mM lactate. 95% confidence limits are indicated. For sulfate reduction rates (SRR), cores were incubated with $^{35}SO_4^{2-}$ for 4 h at 15 to 17°C. Total bacterial abundances were determined by acridine orange staining of bacteria on black polycarbonate filters. Datum points are the means of three counts at each depth. One standard deviation is indicated (error bars).

(diameter, 0.1 to 1 mm), was observed. No trace of macrofauna, except for a few empty small mussel shells surrounded by blackened halos, was observed. At 10 to 12 cm, there was a shift to slightly coarser particles and the appearance of conspicuous amounts of foraminiferans. Throughout the depth, the sediment was composed mainly of ellipsoid pellets, probably fecal pellets, 0.1 to 0.5 mm in diameter. The interstitial volume between the pellets was apparently a free water phase, occasionally filled by a greenish or greyish mass microscopically resembling bacteria in a bacterial exopolymeric matrix. At the time of sampling, salinity in the bottom water was 23‰. The sediment temperature was 9.3°C. The oxygen saturation of the bottom water was not registered. The oxygen penetration was determined to be 2.8 mm in the laboratory under air-saturated water conditions. The porosity of the sediment (ex-

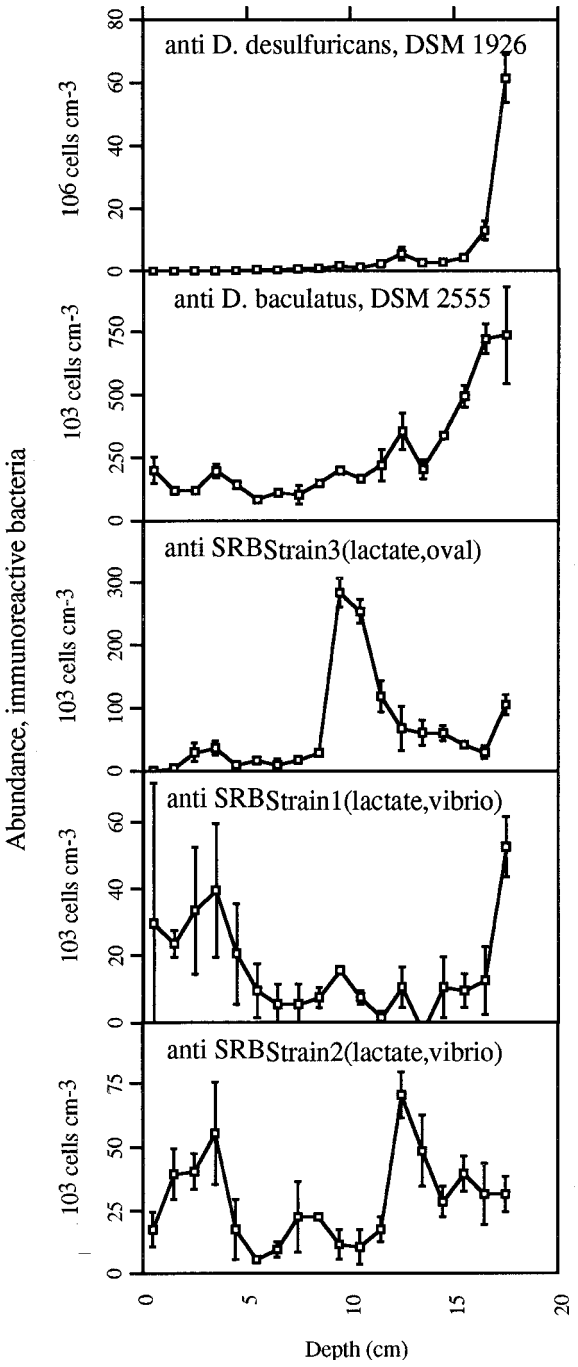

FIG. 2. Depth distribution of immunoreactive bacteria in the sediment. The antisera used are indicated in the panels. Bacteria were counted by indirect immunofluorescence microscopy after reaction with a specific polyclonal rabbit antiserum on black polycarbonate filters and subsequent labelling with a secondary fluorescein-conjugated antibody. Values are means for three filters. One standard deviation is indicated (error bars).

cept for the upper few millimeters) was about 72%. The sulfate concentration dropped from 20 to 9 μmol/cm^3 between depths of 0 and 18 cm. The sediment smelled of H_2S at all depths, except for the top few centimeters. The redox potential dropped from about 0 mV at the surface, exceeding −100 mV at 3 cm and −200 mV at 10 cm.

Sulfate reduction rate depth profile in sediment. The sulfate reduction rate profile is shown in Fig. 1. For the sediment studied, the sulfate reduction rate distribution is typical insofar as it shows a peak of sulfate reduction just below the sediment surface (16). The sulfate reduction rates observed are within the expected range for this type of sediment (16). The maximal rate of 107 nmol cm^{-3} day^{-1} was observed at 1 to 2 cm, and the rate dropped to a minimum of 10 nmol cm^{-3} day^{-1} at 14 to 15 cm but then significantly increased to 38 nmol cm^{-3} day^{-1} in the lower few centimeters.

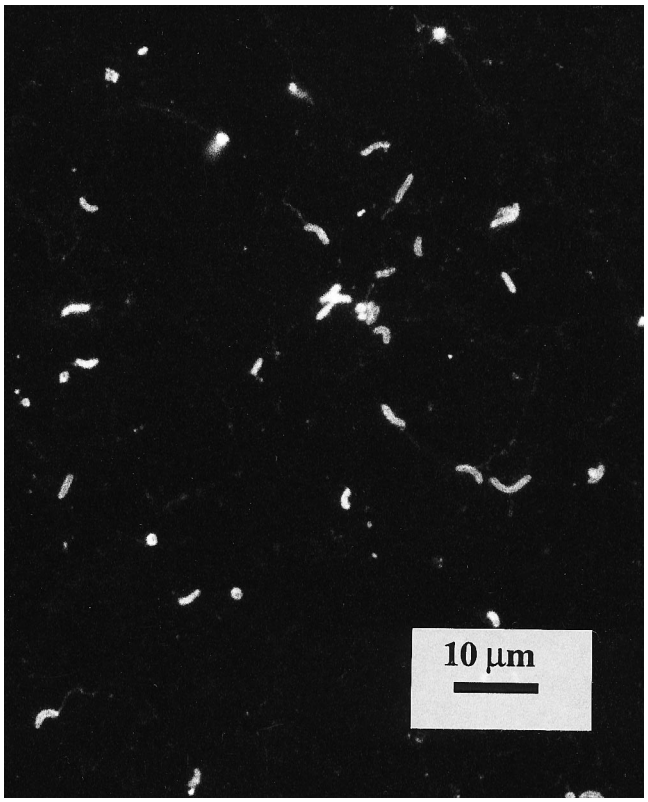

FIG. 3. Microphotograph of immunofluorescent bacteria. Shown is a pure culture of the vibrioid SRB [SRB$_{\text{Strain 1}}$(lactate,vibrio)] stained by indirect immunofluorescence with the homologous anti-SRB$_{\text{Strain 1}}$(lactate,vibrio) serum. Note the staining of the flagella. Size bar, 10 μm. A ×40 objective was used.

Epifluorescence total bacterial counts. The depth distribution of the total bacterial abundance is shown in Fig. 1, with the highest counts of 175 × 10^6 bacteria per cm^3 in the upper few centimeters of the sediment. The bacterial counts then decreased about eightfold. The counts increased in the lower few centimeters of the column to 137 × 10^6, and less-pronounced local maxima could also be observed at 3 to 5, 7 to 8, and 11 to 13 cm.

Viable counts of SRB. The viable counts for SRB grown on lactate are given in Fig. 1. Maximal counts occurred in the 0- to 3.6-cm interval, where counts amounted to 1.5 × 10^5 CFU/cm^3. The counts then rapidly dropped more than 1,000-fold. For the upper three intervals, i.e., down to a depth of 10.8 cm, the dilution series tubes were dominated by motile, vibrioid, and rapidly growing bacteria. In the lower two intervals, bacteria initially grew mainly as films attached to the glass wall and often small vibrioid to rod-shaped bacteria were mixed with larger oval bacteria.

The culture tubes with the higher dilutions were screened for the presence of immunoreactive bacteria by a panel of antisera after 2 and 4 weeks of culture. From the upper two

depth intervals, cultures showed immunological reactions with the anti-$SRB_{Strain\ 1}$(lactate,vibrio) and the anti-*Desulfovibrio baculatus* DSM 2555 sera to a dilution of 10^{-5} [in addition, anti-$SRB_{Strain\ 2}$(lactate,vibrio) serum reacted with one culture at a dilution of 10^{-5} in the uppermost interval], and the 7.3- to 10.8-cm interval showed reaction for anti-*D. baculatus* DSM 2555 and anti-*Desulfovibrio desulfuricans* DSM 1926 sera to a dilution of 10^{-3}. (One or both immunotypes might be present in one tube.) For the 10.9- to 14.4-cm interval, anti-*D. baculatus* DSM 2555 serum reacted to a dilution of 10^{-3} and anti-*D. desulfuricans* DSM 1926 serum reacted to a dilution of 10^{-2}. At 14.5 to 18.0 cm, anti-*D. baculatus* DSM 2555 serum reacted to a dilution of 10^{-3} and anti-*D. desulfuricans* DSM 1926 serum reacted to a dilution of 10^{-2}.

Specificities of antisera. The specificities of the antisera were investigated by immunofluorescence staining of a variety of SRB and non-SRB. Results are presented in Table 1. The antisera used for quantification showed negligible cross-reactions with other sulfate-reducing strains tested, except for anti-*D. desulfuricans* DSM 1926 serum, which reacted with *Desulfobulbus* sp. strain DSM 2058. Negligible cross-reactions were also found for the non-sulfate-reducing strains tested. On the basis of these results, it was not considered useful to absorb antisera for our purposes. The antisera in several cases reacted with sulfate-reducing enrichments and isolates from the Baltic Sea and the North Sea (data not shown).

Immunoreactive bacterial counts. Figure 3 is a microphotograph of immunostained bacteria in pure cultures showing staining of bacteria and flagella. The extracted bacterial fraction of the sediment was screened for immunoreactive bacteria at all depths by the microscope slide technique described above. A panel of 27 different antisera raised against different strains of SRB was used for this screening. For only five antisera were the concentrations of immunoreactive bacteria considered high enough for quantification.

The abundances of immunoreactive bacteria are shown in Fig. 2. For the different antisera, a variety of distributions (unias well as bi- or oligomodal) and densities was observed. An antiserum raised against an SRB strain (*Desulforhopalus* vacuolatus DSM 9700) gave an average count of 7.5×10^3 immunoreactive bacteria per cm^3 of sediment (standard deviation, 5.5×10^3 bacteria per cm^3) and showed no significant peaks throughout the depth. This value was considered due to false positives only, and it was used as a background value and was subtracted from counts obtained for positive sera.

By far the highest counts were observed for the anti-*D. desulfuricans* DSM 1926 serum, but immunoreactive bacteria for this serum were present almost exclusively at a depth of 16 to 18 cm, with a concentration of more than 60×10^6 bacteria cm^{-3}.

Anti-*D. baculatus* DSM 2555 serum showed the highest concentrations, approaching 0.7×10^6 cells cm^{-3}, near the bottom of the core and, in addition, minor increases at 0 to 1, 3 to 4, and 12 to 13 cm.

Anti-$SRB_{Strain\ 3}$(lactate,oval) serum detected one single peak at 9 to 11 cm (284×10^3 cells per cm^3).

Anti-$SRB_{Strain\ 2}$(lactate,vibrio) serum showed several local maxima at 3 to 4, at 7 to 9, and at 12 to 14 cm. The concentration, however, only reached counts of 70×10^3 cells per cm^3.

Anti-$SRB_{Strain\ 1}$(lactate,vibrio) maxima were detected primarily in the upper 0 to 4 cm, and at the very bottom of the core. Anti-$SRB_{Strain\ 1}$(lactate,vibrio) serum gave maximal counts at 2 to 4 cm and values of up to 50×10^3 cells per cm^3.

DISCUSSION

Immunoprobes in microbial ecology. Despite the recognition of marine sediments as widespread habitats for SRB, the present study is to my knowledge the first report (apart from a report of preliminary studies done with phylogenetic probes [6]) on the direct specific observation of SRB in marine sediments.

SRB have previously been studied by immunological methods (1, 23). As a prologue to this discussion, some of the general precautions to be observed when working with immunoprobes are reviewed below. First, immunoreactive bacteria cannot unequivocally be equated to bacteria possessing the physiological trait of sulfate reduction. The antisera have been tested for cross-reactivity against a set of gram-negative (and a single gram-positive) sulfate-reducing bacteria, a panel of strains of sediment or marine origin, and a set of phylogenetically widespread strains. Our data in general support the conclusion that antibodies are strain specific. This was also the conclusion of Smith (24) when studying *Desulfovibrio* strains by immunofluorescence. Except for anti-*D. desulfuricans* DSM 1926 serum, which unexpectedly reacted with the morphologically and phylogenetically different strain *Desulfobulbus* sp. strain DSM 2058, the antisera used for quantification did not cross-react with any of the strains (sulfate reducing or nonsulfate reducing) tested. (In several cases, antisera reacted with new sulfate-reducing isolates and enrichments from the Baltic Sea and the North Sea). The insufficient knowledge of the bacteria inhabiting the marine sediment, however, makes it difficult to draw rigid conclusions from cross-reactivity tests done with cultured strains. SRB isolated from the sediment in our dilution series were in many cases immunoreactive. On the basis of the above-reported results, however, it is justified for present purposes to use the presence of immunoreactive bacteria as an indication of the presence of SRB. The immunoassay does not, in its present form, guarantee that scored bacteria are in fact live and active. This is, however, a drawback shared with most other known techniques, except for methods using mRNA molecules, which have exceptionally short life times, and tetrazolium salt staining, which demands intact electron transport systems.

Bacteria. A prerequisite for the induction of a specific immune response is the ability to obtain the antigen in sufficient quantities. The sources of antigens in this study were bacterial cultures. This assures the physiological assignment "sulfate-reducing bacterium" but, on the other hand, limits the method to cultured organisms or at least to organisms cross-reacting with the cultured strains. According to reference 20, recent environmental isolates often produce useful fluorescent-antibody reagents even when grown on rich laboratory media. Hence, the extent to which the spectrum of cultured strains reflects the actual diversity of actively metabolizing SRB in marine sediments is not known, and bacterial diversity as revealed by rDNA analysis (5) in a marine sediment does point to the possible shortcoming of using only cultured strains. We have in this study limited ourselves to bacteria of temperate marine (sediment) origin and have focused mainly on *Desulfovibrio* strains, but we have also included others, thus covering a wide phylogenetic range of SRB.

Antigenic determinants and specificities of antisera. The antisera stain whole bacteria, and in most cases they preferentially stain the circumference of the bacteria (probably corresponding to cell wall components). Immunoblotting done for some of the antisera indicates that a broad immune response is induced in the rabbits. The strong immunofluorescence signal might, however, be due mainly to a highly repeated unit such

TABLE 1. Specificities of polyclonal antisera prepared against strains of SRB in indirect immunofluorescence reactions

Organism	Result for antiserum						
	Desulfovibrio desulfuricans DSM 1926	*Desulfovibrio baculatus* DSM 2555	$SRB_{Strain\ 1}$	$SRB_{Strain\ 2}$	$SRB_{Strain\ 3}$	*Desulforhopalus vacuolatus DSM 9700*	Nonimmune serum
SRB							
Desulfoarculus baarsii DSM 2075	0	0	0	0	0	0	0
Desulfobacter curvatus DSM 3379	0	0	0	0	0	0	0
Desulfobacter hydrogenophilus DSM 3380	0	0	0	0	0	0	0
Desulfobacter latus DSM 3381	0	0	0	0	0	0	0
Desulfobacter postgatei DSM 2034	0	0	0	0	0	0	0
Desulfobacter postgatei DSM 2553	0	0	0	0	0	0	0
Desulfobacter sp. strain DSM 2035	0	0	0	0	0	0	0
Desulfobacter sp. strain DSM 2057	0	0	0	0	0	0	0
Desulfobacterium autotrophicum DSM 3382	0	0	1	0	0	0	0
Desulfobacterium vacuolatum DSM 3385	2	2	1	0	1	2	0
Desulfobotulus sapovorans DSM 2055	0	0	0	0	0	0	0
Desulfobulbus propionicus DSM 2554	0	0	0	0	0	0	0
Desulfobulbus sp. strain DSM 2058	4	1	1	0	1	0	0
Desulfococcus multivorans DSM 2059	0	0	0	0	0	0	0
Desulfonema limicola DSM 2076	0	0	0	0	0	0	0
Desulfosarcina variabilis DSM 2060	0	1	0	0	0	0	0
Desulfotomaculum orientis DSM 765	0	0	0	0	0	0	0
Desulfomicrobium baculatus DSM 2555	0	4	0	0	0	0	0
Desulfovibrio desulfuricans DSM 1924	0	0	0	0	0	0	0
Desulfovibrio desulfuricans DSM 1926	4	0	0	0	0	0	0
Desulfovibrio gigas DSM 1382	0	0	0	0	0	0	0
Desulfovibrio longus DSM 6739	0	0	0	0	0	0	0
Desulfovibrio salexigens DSM 2638	0	0	0	0	0	1	0
Desulfovibrio sp. strain DSM 2056	0	2	0	0	0	0	0
Desulfovibrio vulgaris DSM 644	1	0	0	0	0	0	0
Desulfovibrio vulgaris DSM 1744	0	1	2	0	0	0	0
$SRB_{Strain\ 1}$(lactate,vibrio)	0	0	4	0	0	0	0
$SRB_{Strain\ 2}$(lactate,vibrio)	ND[a]	0	ND	4	1	0	0
$SRB_{Strain\ 3}$(lactate,oval)	ND	0	ND	0	4	0	0
Desulforhopalus vacuolatus DSM 9700[b]	ND	0	ND	0	0	4	0
Non-SRB, phylogenetic range							
Bacillus subtilis DSM 10	1	1	0	0	0	ND	ND
Comamonas testosteroni DSM 50244	0	0	0	0	0	ND	ND
Escherichia coli DSM 498	0	0	0	0	0	ND	ND
Halobacterium halobium DSM 670	0	0	0	0	0	ND	ND
Micrococcus luteus DSM 20030	0	0	0	0	0	ND	ND
Myxococcus xanthus DSM 435	0	0	1	1	0	ND	ND
Photobacterium phosphoreum DSM 2167	1	2	1	1	1	ND	ND
Pseudomonas fluorescens DSM 50090	0	0	0	0	0	ND	ND
Rhodospirillum rubrum DSM 467	0	0	0	0	0	ND	ND
Non-SRB, marine or sediment origin							
Acetobacterium woodii DSM 1030	0	0	0	0	0	ND	ND
Chlorobium vibrioforme DSM 265	0	0	0	0	0	ND	ND
Chromatium buderi DSM 176	0	0	0	0	0	ND	ND
Methanobacterium sp. strain DSM 3821	0	0	0	0	0	ND	ND
Pseudomonas stutzeri DSM 6084	0	0	0	0	0	ND	ND
Rhodobacter sulfidophilus DSM 1374	0	0	0	0	0	ND	ND
Rhodobacter sulfidophilus DSM 2351	0	0	0	0	0	ND	ND
Rhodococcus marinonascens DSM 43752	0	0	0	0	0	ND	ND
Rhodopseudomonas marina DSM 2780	0	0	0	0	0	ND	ND
Rhodospirillum salexigens DSM 2132	0	0	0	0	0	ND	ND
Spirochaeta litoralis DSM 2029	0	0	0	0	0	ND	ND
Syntrophospora bryantii DSM 3014A	0	0	0	0	0	ND	ND

[a] ND, not determined.
[b] Gift from M. Isaksen; tentatively named (9a).

as a lipopolysaccharide, although reaction with this moiety could not be confirmed by the immunoblotting results in all cases. It is interesting that some of the antisera also reacted with bacterial flagella (Fig. 3). For pure cultures, as well as for environmental samples, immunostaining reveals a single polar flagellum for some of the *Desulfovibrio*-like bacteria. Within the strains tested, flagellar reaction is also specific. Flagella seem to be rather fragile and are often lost after homogenization or strong pipetting.

In addition to showing reaction with the homologous strain,

the antisera raised against *Desulfobacter* strains often showed reactions with other *Desulfobacter* strains (data not shown). This probably reflects the close phylogenetic relatedness of the *Desulfobacter* species.

All antisera showed staining of every cell in the corresponding pure culture. Variation of antigenic properties due to environmental factors might influence detectability of bacteria (17). However, the antigenic determinants in the present case are resistant to a number of physiological changes. For *D. baculatus* DSM 2555, growth at different temperatures (6 to 30°C), low substrate concentration (2 mM lactate), or a lack of sodium chloride does not seem to affect immunofluorescence; neither does growth on different carbon sources (lactate or malic acid) for the vacuolated *Desulfobacterium zeppelini* DSM 9120 (isolated by R. Lillebæk; tentatively named [16a]).

Immunoreactive cells do not necessarily correlate with metabolically active cells, and one major bias may be the contribution of dead or dormant cells to immunoreactive counts.

Sulfate reduction rate profile. Two sulfate reduction maxima were observed, one subsurface and a second at a depth of 16 to 18 cm. Small black inclusions are observed in association with the latter. This might be suggestive of the presence of either detritus particles or meio- or macrofaunic elements. The degradation of such faunal elements could offer an available carbon source for an enhanced population of SRB.

Total bacterial abundances. A mild physical extraction method was applied, and only particles greater than 1 μm with distinct bacterial morphologies were scored. The bacterial counts were within the range reported previously (16) for similar types of sediment. Also, as previously reported for an anoxic-sulfidic sediment of this type, bacterial counts and sulfate reduction rates tend to scale together (Fig. 1).

Viable counts of SRB. The viable counts (MPN) obtained in this study are within the range of those reported previously (29). A more recent work (15) reports high values for H_2- as well as for acetate-utilizing SRB, due in part to a long culture period, which allows the inclusion of slowly growing bacteria in the counting series. In a North Sea sediment, we observed that counts of lactate-utilizing bacteria increased about 10-fold if slow growers were included (personal observation). In the present study, slowly growing bacteria also dominated the higher dilutions. The two cultures obtained at a 10^{-7} dilution in the 0- to 3.6-cm interval were dominated by morphologically *Desulfosarcina*-like organisms (in one of the cultures, small vibrios were also observed). For the lower part of the sediment, poor survival of the slowly growing cultures and detection difficulty due to slow production of hydrogen sulfide are likely to have caused an underestimation of viable counts. As previously reported (29), the numbers of cells are also underestimated if the cells are associated in clumps or attached to particles. The mild homogenization used in this study would make the assay particularly susceptible to this bias.

In contrast to previous reports (15), and our own observations from a North Sea sediment, we did not observe any gas-vacuolated bacteria in this study; neither did we find any sulfate-reducing spirilloid bacteria or other organisms with conspicuous morphologies. A reason for this may be that the upper few millimeters have not been studied in detail.

The viable counts at 17 to 18 cm obtained in this study are low compared with the immunoreactive counts (especially those for anti-*D. desulfuricans* DSM 1926 serum). The strain *D. desulfuricans* DSM 1926 does, however, have a temperature optimum well above the temperature used for the counting series; therefore, the growth conditions may be suboptimal.

Immunoreactive bacterial counts. Some of the reasons accounting for low MPN counts as discussed above may also apply to the immunofluorescence assay. For example, an explanation for the low frequency of members of the family *Desulfobacteriaceae* is that these bacteria often form clumps and are often nonmotile in culture. Indeed, clumps of bacteria were stained with anti-*Desulfobacter* sp. sera in our screening. As the initial screening of the sediment showed only very low numbers of members of the family *Desulfobacteriaceae*, it was only possible to do counts with antisera against strains of *Desulfovibrionaceae*, although the strains of *Desulfobacteriaeae* would have been much easier to recognize because of their larger cells. During initial culture, strains of *Desulfovibrionaceae* may also form bacterial films attaching to glass surfaces. The immunoreactive bacterial counts display several different distribution patterns, indicating that it is of importance to study the ecophysiology of individual strains in order to understand the processes going on in the marine sediment.

Coupling of immunoreactive counts to total bacterial counts. In general, immunoreactive counts and even the sum of all immunoreactive counts are orders of magnitudes lower than total counts. One exception, however, is the immunoreactive counts for anti-DSM 1926 serum. In this case, the immunoreactive counts account for more than half of the total counts in the lowest fraction of the core. Denitrifiers have been studied with immunoprobes (28). The authors of that report conclude that the culturable denitrifier used for antiserum production is a minor component of the natural bacterial assemblage and suggest two explanations for this: (i) culturable strains are inherently unrepresentative of natural populations and (ii) the natural assemblage is composed of many minor components with no numerically dominant strains. Our data suggest that cultivable strains may be the numerically dominating ones under some (yet-unknown) environmental conditions.

Coupling numbers of immunoreactive bacteria to viable counts. In order to investigate the nature of the bacteria growing in the higher dilutions of the dilution series, we screened the cultures with our antiserum panel. We found that in the upper three depth intervals, anti-$SRB_{Strain\ 1}$(lactate,vibrio) serum reacted with almost all tested cultures in the dilution series and anti-*D. baculatus* DSM 2555 serum also reacted with several cultures, while anti-*D. desulfuricans* DSM 1926 and anti-$SRB_{Strain\ 2}$(lactate,vibrio) sera reacted with only a few. This suggests (although it does not prove) that the immunoreactive bacteria detected in the sediment are indeed SRB, that they are cultivable, and, for anti-$SRB_{Strain\ 1}$(lactate,vibrio) serum, that the number of cross-reacting CFU corresponds rather well to the immunoreactive counts obtained. Although anti-*D. desulfuricans* DSM 1926 serum reacts with a few cultures in the lowest depth interval, there is a large discrepancy (orders of magnitude) with the much higher immunoreactive counts obtained. Our data probably do not offer a clear explanation for this discrepancy, but bias introduced during extraction and culturing should be kept in mind.

For the lower two depth intervals, larger oval bacteria reacting with anti-*Desulfobacter postgatei* DSM 2553 serum indicates the presence of *Desulfobacter* spp. in the cultures. As none of the *Desulfobacter* spp. are reported to grow on lactate, they are probably present as secondary cocultures feeding on acetate produced by incompletely oxidizing SRB.

Conclusion. The immunofluorescence method developed in this study allows us to quantify bacteria by using anti-SRB sera in marine sediments. In order to detect the small population sizes, great care must be taken in optimizing extraction, staining, and counting techniques. In the present study, it has become clear that there are several different distribution patterns of immunoreactive bacteria. The different patterns suggest that

different SRB strains occupy niches separated either physically or by temporal succession. Seasonal variations, diurnal migrations, chemotaxis, spatial organization, and symbiosis should eventually be addressed by the technique. In combination with hybridization and oligonucleotide probe studies of genomic and messenger sequences (e.g., NiFe hydrogenase in *Desulfovibrio* spp.) (26) specific for SRB, the immunofluorescence technique could become a cornerstone in microbial ecological studies of SRB.

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