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Dominant Marine Bacterioplankton Species Found among Colony-Forming Bacteria

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The density of specific aquatic bacteria was determined by use of whole-genome DNA hybridization towards community DNA. From a coastal marine environment (northern Baltic Sea), 48 specific bacteria were isolated on solid media over a 1-year period. Based on the presented hybridization protocol, the total density of the isolates ranged between 7 and 69% of the bacteria determined by acridine orange direct counts. When compared to the number of nucleoid-containing cells, the range increased to 29 to 111%. Thus, our results showed that bacteria able to form colonies on solid media accounted for a large fraction of the bacterioplankton. There were significant changes in the density of the different bacteria over the year, suggesting that bacterioplankton exhibit a seasonal succession analogous to phytoplankton. The bacteria studied were of diverse phylogenetic origin, being distributed among the α, β, and γ subdivisions of the class *Proteobacteria* and the cytophaga-flexibacter group. Partial 16S rRNA gene sequence analysis of 29 Baltic Sea isolates as well as of 30 Southern California Bight isolates showed that a majority of the isolates had low similarity (0.85 to 0.95) to reported sequence data. This indicated that the diversity of marine bacteria able to grow on solid media is largely unexplored.

In spite of the importance of bacterioplankton in the global carbon cycle, few reports have demonstrated the variable activities of specific heterotrophic bacteria. Instead, bacterioplankton are usually clumped together into a "black box" in conceptual and mathematical models. This anonymity is unwarranted since different marine bacteria, grown under the same conditions, exhibit different ectohydrolytic enzyme profiles, suggesting that different bacteria degrade different fractions of organic matter in the sea (30). Thus, further insight into species density and distribution of marine bacteria would be desirable. A multitude of marine bacteria have been isolated during the last century (11). Yet, at present, a largely unconfirmed opinion claiming that these isolates may not be representative of the indigenous bacteria persists. Therefore, most recent reports on bacterial communities are concerned primarily with the number of species potentially present in different habitats (3, 5, 10, 14, 21, 43, 48), and only a few reports concern the actual abundance of specific bacteria or their impact on the environment (17, 41, 49).

The view that marine isolates are not representative of the indigenous bacteria stems from the unexplained disagreement between plate counts and acridine orange direct counts (AODC), and it has become the current dogma that marine bacteria are unculturable (22, 25). Hence, culture-independent PCR-based methods have been used to obtain bacterial gene libraries from the sea. This work has revealed 16S rRNA sequences of previously unsequenced and possibly uncultured "new" bacteria (13, 16, 35, 43). In these studies, isolated bacteria serve only as references for homologous 16S rRNA sequences obtained from the cloned gene libraries (36). At the same time, data demonstrating the dominant occurrence of culturable bacteria in the sea have been reported. With Southern California Bight isolates, Rehnstam et al., using 16S rRNA probes, showed that single species of culturable bacteria can dominate the community DNA (40). Moran et al. showed that genus-specific 16S rRNA probes from cultured bacteria accounted for 5% of the bacterial community in a coastal sediment based on microscopy count (33). Also, Fuhrman et al. have reported that two culturable marine isolates each accounted for up to 20% of the bacterial community (12). These isolates were collected, one from the Caribbean Sea and one from the Pacific Ocean, and detected by the use of total genomic probes. Voordouw et al. have developed and used a protocol for quantitative reverse sample genome probing of microbial communities based on whole-community DNA probes (47). These authors have shown clear changes in the composition of the bacterial community with time. This exciting development in marine microbiology makes it possible to open the bacterial black box and to determine bacterial diversity and annual bacterial succession in a specific sea area.

To assess the absolute abundance of specific bacteria in seawater samples, we have developed a species density protocol, involving the use of whole-genome DNA hybridization towards community DNA, which consists of three steps: sampling of bacterial community DNA, preparation and labeling of genomic DNA from cultured isolates, and preparation of a standard curve for each isolate relating bacterial numbers to hybridization signal. With the aim to describe the bacterial community, we determined the abundance of the indigenous bacteria and analyzed the diversity of the isolated bacteria based on partial 16S rRNA gene sequences.

MATERIALS AND METHODS

Sampling. The study area was NB1, a routine sampling station in the northern Baltic Sea (63°30′N, 19°48′E). Based on annual averages of primary production, chlorophyll *a*, and bacterial production, this station is representative of a coastal marine ecosystem (1, 8, 50). Seawater samples for bacterioplankton community DNA were collected at a depth of 4 meters on 10 occasions during the entire sampling period (6 April to 18 October 1995). Seawater (20 liters) was collected at each sampling occasion in a carefully acid-rinsed polycarbonate bottle (with a large volume to decrease perturbations due to handling). The seawater was kept at in situ temperature, and samples were filtered for bacterial counts and hybridization (cells lysed and DNA linked to the membrane) within 4 h after

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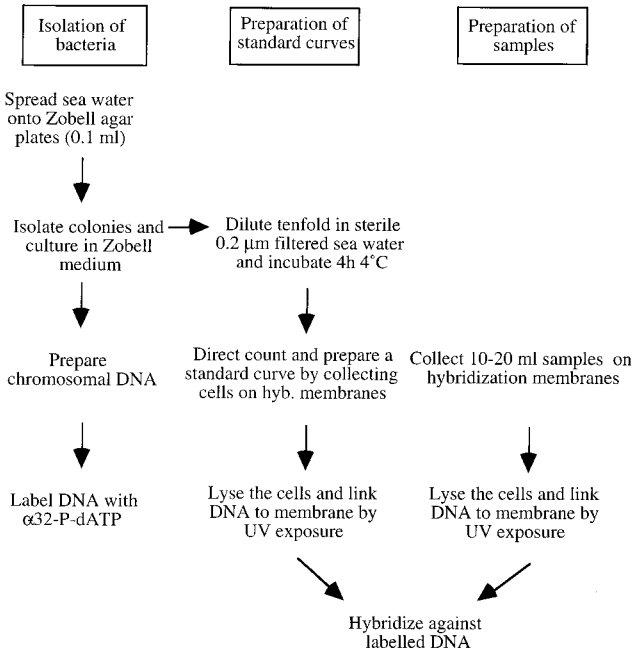


FIG. 1. Outline of the major steps in the species density protocol, by use of whole-genome DNA hybridization towards community DNA.

sampling. In addition, bacterial isolates for comparison of species diversity in a second marine environment were obtained from the Southern California Bight (32°53′N, 117°15′W) in December 1995.

Enumeration of bacteria. Total counts of bacteria were determined by fluorescent staining by filtering the cells onto 0.2-μm-pore-size black filters (Micron Separations, Inc.) and staining the cells for 5 min with a 0.003% acridine orange solution essentially as described for AODC (20). Numbers of nucleoid-containing bacterial cells (NUCC) were quantified by the DAPI (4′,6-diamidino-2-phenylindole) staining-destaining protocol described by Zweifel and Hagström (55). The number of CFU was determined by plating on Zobell agar plates (53). Plates were incubated in the dark at 15°C until no more colonies appeared (within 10 to 15 days).

Species density protocol. The major steps of the species density protocol are outlined in Fig. 1. From the agar plates used for determination of CFU, isolates were obtained for cultivation in Zobell medium at 15°C. Chromosomal DNA from the isolates was prepared essentially as described by Wilson (51). The DNA was resuspended in 100 μl of water at 4°C for at least 24 h. To remove RNA residues, 10 to 100 μg of RNase A (Sigma) per ml was added and the sample was incubated for 30 min at 37°C. DNA yield was determined by agarose gel electrophoresis and comparison to known concentrations of DNA. Labeling of the chromosomal DNA for use as probes was performed with a nick translation kit (Promega) and [α-^{32}P]dATP (Amersham), giving a specific activity of 0.1×10^8 to 1×10^8 cpm/μg. Probes were purified from free nucleotides with spin columns (Pharmacia).

Standard curves for single bacterial species were prepared by culturing isolates in Zobell medium (53) at 15°C and harvesting the bacteria in known numbers onto hybridization membranes. Cultures were grown for 1 to 2 days (approximately 10^8 cells ml^{-1}) and thereafter 10-fold diluted with sterile-filtered (0.2-μm pore size), autoclaved seawater from the area where the bacteria were originally isolated and kept at 4°C for 3 h before enumeration by direct counts and subsequent filtration. Four replicates of each standard curve with 0.5×10^5 or 1×10^5, 3×10^5, 6×10^5, 1×10^6, 2×10^6, or 5×10^6 cells in a final volume of 10 ml were filtered onto six dots simultaneously. The cells were lysed on the hybridization membranes as described for the seawater samples. The standards for each isolate were hybridized with the corresponding whole-genome probe in the same hybridization flask as that used for the community DNA samples covering the whole sampling season.

Preparation of bacterial community DNA from the seawater samples was performed as follows. Seawater samples were prefiltered through a 2-μm-pore-size filter (MSI Polycarbonate). We recommend prefiltration to reduce clogging of the hybridization membranes, since 2-μm-pore-size filtrations do not reduce the number of bacteria. The samples were then filtered onto 0.45-μm-pore-size hybridization membranes (Hybond-N; Amersham) with a blotting apparatus (GIBCO BRL; with 6-mm-diameter slots) with a modified lid containing 24 10-ml wells, allowing larger sampling volumes. Ten to 20 ml of seawater was filtered at 200 to 300 mm Hg. Less than 0.1% of the bacteria were found to pass through the membranes (data not shown). The low number of bacteria that pass through the membranes is explained by the property of the nylon membrane. It effectively retains particles much smaller than the nominal pore size of the membrane due to the fiber structure of the membrane. The pore size indicates only the maximum-sized particles that will pass through the membrane. This is in contrast to polycarbonate filters that act like sieves with holes of uniform size, based on the nominal pore size. The samples were lysed in the dot blotting apparatus essentially as described by the membrane manufacturer (Amersham). The slot was covered with 100 μl of 0.5 M NaOH for 3 min, the solution was filtered through, and the procedure was repeated. The slot was covered with 100 μl of 1 M Tris-HCl (pH 7.4) for 5 min, the solution was filtered through, and the procedure was repeated. Finally, the slot was covered with 100 μl of 1.5 M NaCl–0.5 M Tris-HCl (pH 7.4) for 5 min, and the solution was filtered through. The membrane was placed on Whatman 3MM paper and air dried for 15 min. The DNA was then linked to the membrane by optimal cross-linking (1,200 × 100 μJ per cm^2, 14 s) in a XL-1000 UV cross-linker as described by the manufacturer (Spectronics Corporation). Alternatively, the lysis protocol of Amersham, i.e., placing the membranes directly in the solutions on Saran Wrap, was also used. The membranes were stored at 4°C until analysis.

The following hybridization conditions were used (28). The membranes were prehybridized in a solution consisting of 10× Denhardt solution (50× Denhardt solution, 1% Ficoll, 1% polyvinyl pyrrolidone, and 1% bovine serum albumin, 4× SSC (20× SSC is 3M NaCl plus 0.3 M Na citrate), 0.1% sodium dodecyl sulfate, 2 mM EDTA, and 100 μg of salmon sperm DNA per ml for at least 2 h at 69°C with a hybridization incubator (Robbins Scientific). Two micrograms of the probe was denatured in boiling water for 10 min and added to the hybridization solution. The membranes, community DNA samples, and standard for each isolate were hybridized in the same hybridization flask overnight at 69°C. The membranes were washed twice, 30 min each time, in 2× SSC–0.5% sodium dodecyl sulfate at the hybridization temperature and twice, 5 min each time, in 0.1× SSC at room temperature. The membranes were wrapped in Saran Wrap and exposed on a PhosphorImager (Molecular Dynamics) for detection of the hybridization signal. The relationship between hybridization signal and number of bacteria was obtained from the slope of the standard curve for each isolate.

Cross hybridization between isolates was determined by hybridization of extracted DNA. Blotting of the DNA was as described by the membrane manufacturer (Amersham). The DNA extracts were diluted in 10× SSC and denatured at 95°C for 5 min, transferred to ice, and thereafter filtered onto hybridization membranes (Hybond-N; Amersham) with a slot blot apparatus (GIBCO BRL), after which the DNA was denatured, neutralized, and linked to the membrane as described above for sample preparation. Hybridization was performed as described above.

PCR amplification, purification of PCR product, and sequencing. DNA coding for 16S rRNA was amplified by means of PCR with *Taq* polymerase (Boehringer-Mannheim) from DNA preparations of cultured isolates. Bacterial 16S rDNA primers, 27f:biotinylated (AGAGTTTGATCATGGCTCAG) and 1492r (TACGGYTACCTTGTTACGACTT), were used for amplification (15). The reaction volumes were 50 μl, containing 1 μg of template, 10 mM total deoxynucleoside triphosphates, standard 10× *Taq* buffer, a total of 15 ng of each primer, and 1 U of *Taq* polymerase. The PCR was carried out for 30 cycles, with each PCR consisting of one round of denaturation at 95°C (2 min), annealing at 50°C (30 s), and elongation at 72°C (45 s), and then for 29 cycles of 95°C (30 s), 50°C (30 s), and 72°C (45 s) with a DNA thermal cycler 480 (Perkin-Elmer). The biotinylated strand was purified with streptavidin-coated magnetic beads (Dynabeads M280-Streptavidin; Dynal AS, Oslo, Norway). Twenty-five microliters of Dynabeads was washed once in TES (Tris-EDTA plus 0.1 M Na Cl), resuspended in 50 μl of TES, and incubated for 30 min with the 50-μl PCR mixture at room temperature. The biotinylated strand was purified by denaturation for 5 min with 100 μl of 0.15 M NaOH and washing once with TES and once with water. The Dynabeads with the purified biotinylated strand were resuspended in 11 μl of water. Nucleotide sequences were determined from the purified single-stranded 16S rDNA by automated sequencing with an ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer) with primer 518r (CGTATTACCGCGGCTGCT) (27).

Phylogenetic analysis. The phylogenetic tree was calculated by a parsimony method by use of PAUP 3.0 for the MacIntosh. The tree was calculated from the sequences corresponding to nucleotide positions 66 to 506 (*Escherichia coli* numbering) and rooted with the gram-positive *Microbacterium arborescens* as an outgroup. Bootstrap values larger than 50% supporting the branches, as obtained from 100 resamplings, are indicated at the branches. Phylogenetic trees calculated by a distance matrix method (UPGMA) yielded a similar topology. Comparison of sequences of the isolated bacteria to data bank sequences (EMBL) was made for the same nucleotide positions as those used for the phylogenetic tree, comprising both variable and conserved regions. Sequences of bacteria containing multiple ambiguous positions or sequences not overlapping the whole region of comparison were omitted from the analysis.

Nucleotide sequence accession numbers. Sequences from the isolates reported here have been submitted to GenBank. The designation BAL was used for isolates collected from the Baltic Sea, and SCB was used in designations for isolates collected from the Southern California Bight. Sequences for the following isolates were filed under the accession numbers indicated: BAL2 to -5,

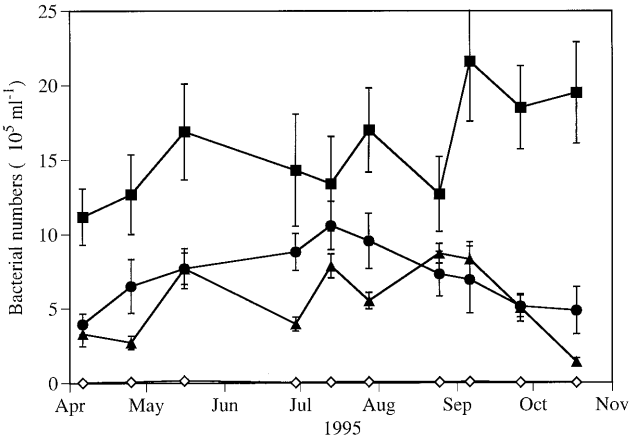


FIG. 2. Total density of the 48 species of bacteria (▲) compared to AODC (■), number of NUCC (●), and CFU (◇). Error bars indicate standard deviations (n = 3, 15, 15, and 6 for species density, AODC, NucC, and CFU, respectively). The CFU ranged from 0.017×10^5 to 0.18×10^5 per ml during the sampling period. The density of the 48 species was determined by the species density protocol with bacterial community DNA samples collected at NB1 (63°30′N, 19°48′E) during 1995.

U63934 to -37; BAL9, U63938; BAL11, U63939; BAL13, U63940; BAL15 to -19, U63941 to -45; BAL22, U63946; BAL23, U63947; BAL25, U63948; BAL27, U63949; BAL29, U63950; BAL31, U63951; BAL34, U63952; BAL37 to -40, U63953 to -56; BAL43 to -48, U63957 to -62. Sequences for the isolates SCB21 to -50 were filed under accession numbers U63998 to U64027.

RESULTS

Abundance of specific bacteria in seawater. During the sampling period 1995, bacteria isolated on solid media and bacterial community DNA were collected on 10 occasions from the routine sampling station NB1 in the northern Baltic Sea. Bacterial isolates were then subjected to colony hybridization to weed out duplicates (40). The abundance of the resulting 48 unique isolates was thereafter determined by the species density protocol, by hybridization of whole-genome DNA probes towards community DNA. Bacteria able to form colonies on solid media made up a significant fraction of the Baltic Sea bacterial community on most sampling occasions (Fig. 2). From April through October, the total density of bacteria that could be obtained by hybridization of the 48 species-specific probes corresponded to 7 to 69% of the counts determined by the AODC. However, several studies have indicated that a large fraction of the particles counted as bacteria by traditional staining procedures, such as AODC, consist of empty cells. These methods include transmission electron microscopy of sectioned bacteria, in situ hybridization to rRNA, and fluorescent staining specific for DNA (6, 19, 23, 55). Thus, relating numbers obtained by hybridization to AODC may underestimate the fraction of the bacterial community accounted for by the isolated bacteria. When the numbers were instead compared to the number of NUCC, the density of the 48 isolates accounted for 29 to 111% of the cells (Fig. 2).

Cross hybridization of more than 5% was found in less than 3% of the possible cases (n = 595, based on the 35 most common isolates). In Table 1, the densities of the different species present on 25 August are shown. Among the 32 isolates detected on this date, the abundance of the 13 most numerous isolates accounted for 86% of the NUCC while the remaining 19 isolates accounted for only 14%. The maximum error due to cross hybridization was calculated to be 13.5% on this occasion. This value was obtained by correcting for cross hybridization between all species occurring on the sampling occasion. The pattern of species distribution, with a few dominant species, was consistent throughout the sampling period (data not shown). A majority of the isolates detected displayed changes in abundance with time of the year (Fig. 3). The annual distribution of a few isolates can illustrate this point. The isolates

TABLE 1. Abundance of the specific bacteria present at NB1 on 25 August 1995

Isolate	Scientific name	Abundance (mean ± SE [10^4] ml^{-1})	% of total
BAL46	*Sphingomonas* sp.	16.4 ± 1.54	18.9
BAL3	*Caulobacter* sp.	13.0 ± 0.56	15.0
BAL29	*Flectobacillus* sp.	5.8 ± 0.25	6.7
BAL37	*Alcaligenes* sp.	5.6 ± 0.09	6.4
BAL11	*Rhizomonas suberifaciens*	5.3 ± 0.38	6.1
BAL44	*Flavobacterium* sp.	4.9 ± 0.04	5.7
BAL18	*Pseudomonas fluorescens*	4.3 ± 0.21	4.9
BAL33	*Sphingomonas yanoikuyae*	4.2 ± 0.18	4.8
BAL17	*Cytophaga* sp.	3.7 ± 0.20	4.2
BAL41		3.4 ± 0.17	3.9
BAL9	*Flavobacterium* sp.	3.2 ± 0.25	3.7
BAL31	*Pseudomonas* sp.	2.7 ± 0.13	3.1
BAL35	*Sphingomonas* sp.	2.1 ± 0.04	2.4
Others[a]		12.5 ± 0.35	14.4
Total		87.2 ± 1.8	100

[a] Includes 19 isolates, each comprising <2% of total.

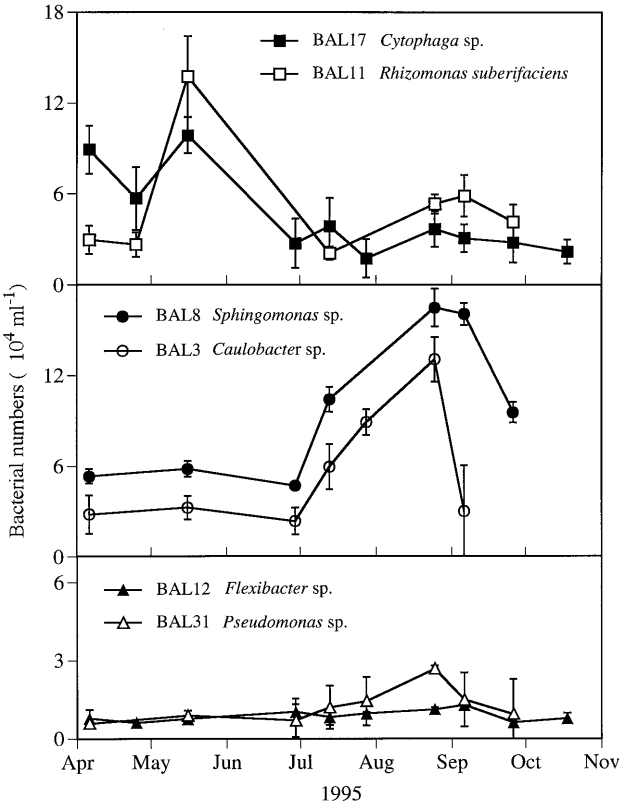


FIG. 3. Seasonal dynamics in abundance of selected bacterial species in the Baltic Sea during 1995, determined by the species density protocol. Error bars indicate standard deviations (n = 3).

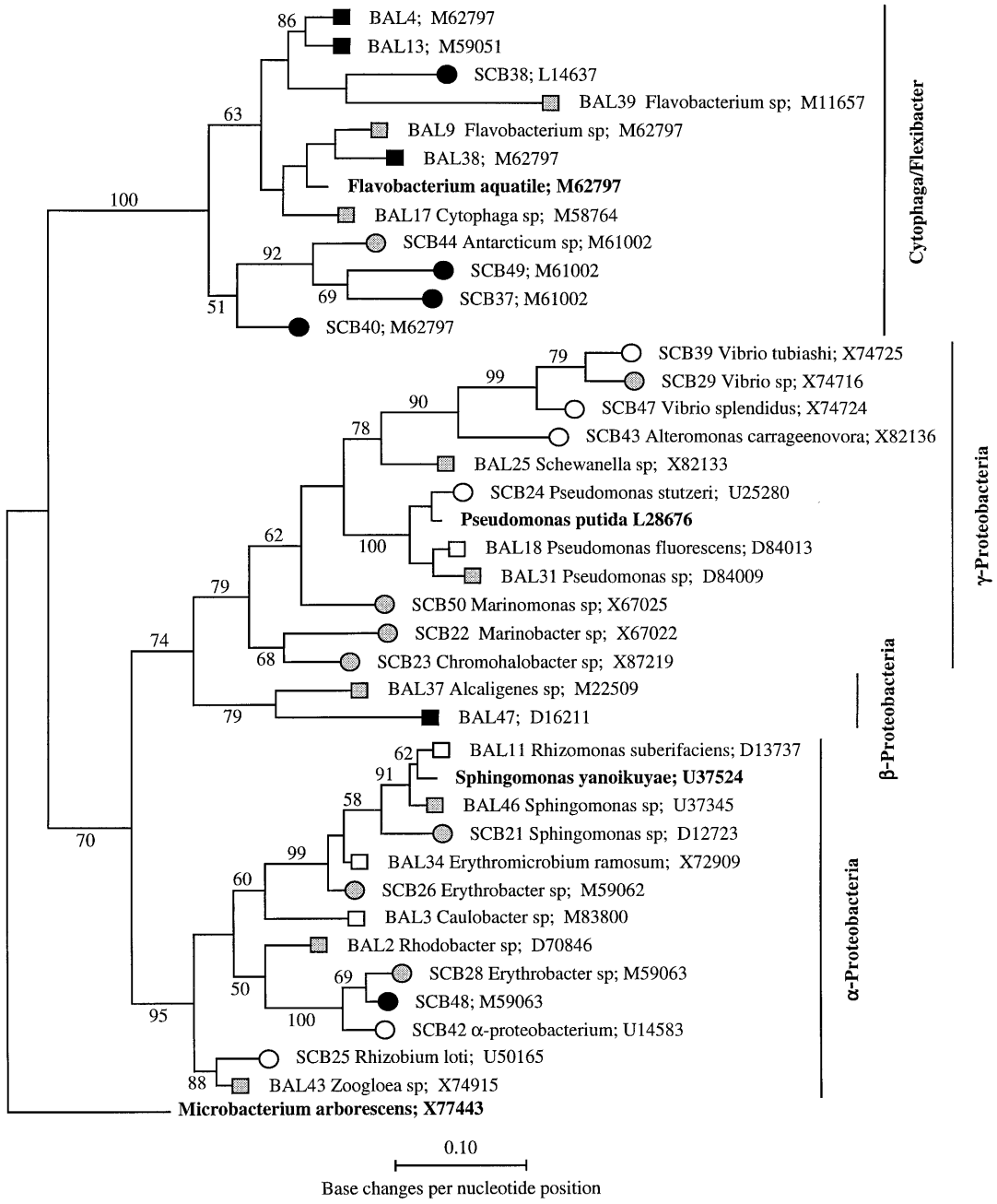
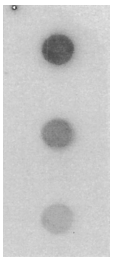


FIG. 4. Phylogenetic tree illustrating the relationship among partial 16S rDNA sequences of bacteria isolated on solid media from the northern Baltic Sea and the Southern California Bight and organisms from the EMBL sequence data bank (boldface). Similarity values of the sequences of our isolates to EMBL data bank sequences are indicated by shading of symbols preceding the BAL (squares) and SCB (circles) isolate designations. Species with similarity values of >0.97 were considered to be identical species and hence the designated scientific name is presented (□, ○); species with values of >0.93 and <0.97 assume identity on the genus level (▨, ◍); species with values of <0.93 represent a species with uncertain classification and thus the scientific designation is omitted (■, ●). The isolate name is followed by the scientific name derived from the closest matching organism identified by its accession number.

BAL17 (*Cytophaga* sp.) and BAL11 (*Rhizomonas suberifaciens*) dominated during spring, while BAL8 (*Sphingomonas* sp.) and BAL3 (*Caulobacter* sp.) dominated in autumn. Isolates BAL12 and BAL31 represent isolates that were present in low numbers throughout the year.

Species composition. Sequencing partial 16S rRNA genes of 29 of the most frequent Baltic Sea isolates revealed only 7 isolates with sequence similarity values higher than 0.97, 16 isolates with similarities ranging from 0.93 to 0.97, and 6 isolates with similarities ranging from 0.86 to 0.93 to previously characterized bacteria in the EMBL data base (Fig. 4). Sequences of additional marine bacterial isolates collected in the Southern California Bight revealed a similar range of 16S rRNA similarity values when compared to data base sequences. Of 30 Southern California Bight isolates, 6 had sequence similarities higher than 0.97, 12 had similarities ranging from 0.93 to 0.97, and 12 had similarities ranging from 0.85 to 0.93 to previously characterized marine bacteria. The se-

TABLE 2. Direct hybridization of isolate SKA17 towards a mixed bacterial community[a]

Sample vol (ml)	PhosphorImager	Corrected counts[b]	No. of SKA17 in sample (10^5)[c]	Concn of SKA17 (10^4 ml^{-1})[d]
9		33,700	3.09	3.43
4.5		17,100	1.57	3.48
1.5		5,800	0.53	3.53

[a] The total number of bacteria in the sample was 2.7×10^5 ml^{-1}.
[b] Volume count was measured with a PhosphorImager (Molecular Dynamics), and corrections were made for exposure time (265-min standard/1,335-min sample).
[c] Counts multiplied by 9.17 as determined from the slope of the standard curve for SKA17.
[d] Mean concentration of SKA17 (± standard deviation), $(3.48 \times 10^4 \pm 0.05 \times 10^4)$ ml^{-1}.

quenced bacteria were distributed among three major groups of bacteria (Fig. 4), the α and γ subdivisions of the class *Proteobacteria* (α- and γ-Proteobacteria), and the cytophaga-flexibacter group, of which the first two are routinely detected by cloning techniques (14, 16, 35, 43). Only two members of the β-Proteobacteria were found, and finally three bacteria, SCB22, SCB23, and SCB50, were found to branch deeply among the γ-Proteobacteria.

Accuracy of the species density protocol. The standard curves were linear over the range tested (5×10^4 to 5×10^6) with a r^2 in all cases higher than 0.96 (linear regression, $P < 0.001$). Extrapolating the standard curves, the absolute deviation from zero was $1.9 \times 10^4 \pm 2.1 \times 10^4$ cells ($n = 48$). The detection limit can be designated to be three times the standard deviation, in this case, 6.3×10^4. This means that a specific bacterium that represents ≥0.5% of the bacterial community can be detected in 10 ml, assuming an ambient number of bacteria of 10^6 ml^{-1}. The effect of sample volume on the hybridization signal from one specific species was tested in a mixed bacterial community. With isolate SKA17 as a probe, the sample volume was varied sixfold with no obvious effect on the hybridization efficiency and on the resulting number of cells (Table 2). This result was consistent for all eight isolates tested (data not shown). The isolate SKA17 is a previously undescribed bacterium with a sequence similarity of 0.90 to *Methylosinus sporium*, accession number M95665 in the EMBL sequence data bank. To further investigate the unspecific background contribution by free dissolved DNA in particle-free seawater, bacteria of the Skagerrak isolate SKA33 (collected in July, sequence similarity of 0.93 to *Sphingomonas* sp., accession number X87167) were added in a known number to filtered (0.2-μm pore size) seawater collected from the Skagerrak in three different seasons (April, July, and September). Between seasons, there was only a 3% offset in the slope of the standard relating hybridization to bacterial numbers.

DISCUSSION

Using the species density protocol, we have determined the abundance in situ of specific bacteria in the northern Baltic Sea over 1 year. This was done by labeling the genomic DNA from unique isolates for use as probes. The genomic probes were then hybridized to community DNA of indigenous marine bacteria lysed on membranes. Standard curves with a known number of cells, lysed under identical conditions as the natural samples, were used to enumerate the isolated bacteria in the natural community. This approach is similar to that used by Voordouw et al. in which labeled extracted community DNA was hybridized to genomic DNA prepared from different bacteria and immobilized on membranes (47). Their protocol allowed the determination of the relative abundance of different bacterial standards in the community DNA. However, since in our protocol both the natural samples and the standards were lysed directly on the membranes in a blotting apparatus (avoiding separate DNA extraction), a direct translation of hybridization signal to bacterial numbers was conceivable. Our results showed that the abundance based on hybridization of the isolated marine bacteria was significantly higher than the total CFU. A total of 48 isolates accounted for a substantial fraction of the bacterial community.

An important concern of the present study was the specificity of the genomic probes and to what degree cross hybridization between species could have influenced our estimates of bacterial numbers. Palleroni et al. reported that DNA-DNA hybridization has a high resolution for closely related organisms (37). They found 0% DNA-DNA homology between *Pseudomonas* species although DNA-rRNA homologies ranged from 80 to 95%. Other studies demonstrate the same usefulness of DNA-DNA hybridization for distinguishing related organisms at the species level (22, 28). In our hands, the isolates with available 16S rDNA sequence homology (69 to 97%) showed a cross hybridization of 2.5% ± 9.8% ($n = 120$). To evaluate the effect of this cross hybridization on the abundance of the 48 isolates, we compiled the maximum cross hybridization for one sampling occasion (25 August). This resulted in a possible overestimation of the abundance of 13.5%, contributed by cross hybridization.

A second concern about the accuracy of the protocol might be the possible interference from free dissolved DNA. However, if free dissolved DNA did contribute to the hybridization signal in the samples, it would also, at least occasionally, have caused a significant background (y intercept) in the standard curves since filtered seawater was used to dilute the cultures. This was not the case, although ambient concentrations of dissolved DNA in the Baltic Sea have been reported to be in excess of 10 μg liter^{-1} (46). The absence of hybridization signal in spite of the presence of free DNA can be understood if the pool of newly released free dissolved DNA has a rapid turnover. Turk et al. reported a high DNA turnover in the Baltic Sea of around 6% of the added tracer per hour (46). Thus, we consider the impact of free dissolved DNA on the estimated abundances to be minor but suggest that cell-free controls be included in future applications of the protocol.

The variable DNA per cell content of the cultured isolates versus the in situ DNA per cell content also represents a considerable source of uncertainty. *E. coli*, however, when growing at 37°C with a generation time of 1.25 h, has 1.5 genome copies (26). Ryals et al. have shown that the macromolecular cell composition is temperature independent and that the Q_{10} for growth rate, RNA chain elongation, and peptide chain elongation is close to 2 (42). Thus, an *E. coli* growing at 15°C would have a generation time of 5 h and still have the same number of genome copies (4, 7). Since our standard curves were made from cultures grown in nutrient broth at 15°C, with generation times exceeding 5 h, we assume that the bacteria used for standard curves carry no more than 1.5 copies of the chromosome per cell. If the genome copy number for the bacterial standards was significantly higher than that for

bacteria in the sea, the abundance of the isolated bacteria in the bacterioplankton would be underestimated. Thus, according to general bacterial physiology, a conservative conclusion is that the density of the different bacteria could be determined with a sufficient precision to name the dominant species on each sampling occasion.

During midsummer and late autumn, only between 20 and 50% of the NUCC could be accounted for by the probes (Fig. 2). Possibly, some species dominating at these periods were overlooked in the selection of isolates or represent species unable to grow on the medium used. The latter explanation is associated with the since-long-observed disagreement between AODC and CFU counts in seawater. Being 3 orders of magnitude and greater, this inconsistency has been used as an argument for why bacteria forming colonies on solid media might be an insignificant fraction of the bacterioplankton (14, 16, 48). A possible explanation for this discrepancy was provided by Rehnstam et al., who suggested that low plating efficiency was due to the extensive viral infection of marine bacteria. In natural samples, the fraction of marine bacteria infected with virus may be as high as 50% of the AODC (31, 39). Moreover, results from transmission electron microscopy, autoradiography, and counting of NUCC and of actively respiring cells have shown that the effective population size is considerably lower than the AODC (6, 19, 29, 32, 55). Thus, a majority of the active cells in the sea may be moribund due to virus infection, and this provides an explanation for the low plating efficiency in natural samples.

Rehnstam et al. also compared the abundance of specific bacteria by 16S rRNA probing of the bacterial community DNA and by colony hybridization with the same probe. Their results showed no relationship between the strength of the hybridization signal towards the community DNA and the CFU (40). This is conceivable since virus infection depends on a numerical response between bacteria and viruses. Thus, there is no reason to assume that species dominating the bacterial community DNA also should dominate the CFU. A second explanation for the plate count anomaly may be senescence of bacteria due to stress, but this too would be a random occurrence, and no correlation between CFU and species distribution should be expected (54).

When the species composition was examined, it was striking that a limited number of species consistently represented a majority of the bacterial community on each sampling occasion. In a high-diversity community, a majority of the species present would be found in approximately equal densities (52). Thus, the bacterial community in this study could be considered moderately diverse since less than half of the species detected on each sampling occasion constituted a majority of the numbers (86% of the NUCC on 25 August). Some of the most abundant species (BAL3, -8, -11, and -17) showed significant seasonal variation. Similar temporal changes in species occurrence were found by Rehnstam et al. at a sampling site off of Scripps Pier, showing that species present in December were undetectable in spring and early autumn (40). Also, Lee and Fuhrman compared bacterial communities in the Pacific in June and November, observing a low similarity between sampling occasions (28). For phytoplankton communities, several biotic and abiotic factors are thought to act in concert to structure the community in a seasonal succession. The annual changes in abiotic factors such as light regime, temperature, and/or other physical disturbance determine the boundaries within which the biotic factors (such as competition, predation, and infection) can regulate the response of different populations (2). The same argument is likely true for the succession of bacterial species in which those species present in low concentrations represent the potential of the bacterial community to respond to future changes (18).

The isolated and sequenced bacteria belonged to widely different bacterial phylogenetic groups, as indicated by the distribution of isolates among different subclasses of the class *Proteobacteria* and the cytophaga-flexibacter division. To compare the Baltic Sea isolates with bacteria from a second marine environment, isolates from the Southern California Bight were included in the phylogenetic tree. We have assumed that 0.97 and 0.93 represent levels of identity at the species (35) and genus levels, respectively. By using these criteria, a majority of our isolates belonged to new species within already-characterized genera, with several isolates possibly representing previously undescribed genera of marine bacteria. Bacteria isolated from both sea areas showed similar phylogenetic distributions and taxonomic resolutions. The poor match of the sequences of isolated bacteria to data bank sequences parallel the findings obtained by cloning amplified 16S rRNA genes from community DNA (14, 16, 35, 43, 48). This observation is interesting since it suggests that due to lack of sequence data from bacteria in culture collections, conclusions that most clones represent new unculturable bacteria may be premature (35). Mullins et al. comment that the γ-Proteobacteria subclass contains the largest number of sequences from cultured marine isolates (35). This is reflected by the high degree of similarity between our sequences and that of members of the genera *Vibrio*, *Alteromonas*, and *Pseudomonas* and data base sequences. Among the isolate sequences matching sequences from the cytophaga-flexibacter group, the degree of similarity to data base sequences was low, with several sequences showing only 0.85 similarity. Thus, this division may contain several unknown genera. Bacteria associated with the cytophaga-flexibacter group were found in a higher proportion in the Baltic Sea than in cloned material elsewhere (14), which possibly reflects the terrestrial influence in the coastal Baltic Sea, since *Cytophaga* spp. are known to degrade complex macromolecules, i.e., lignin and cellulose (45). Caulobacters are known for their ability to survive and grow in water with a low organic content and are thus thought to be important in oligotrophic environments (38); their peak occurrence coincided with the late summer clear-water period. The isolates SCB22, -23, and -50, representing *Marinobacter*, *Chromohalobacter*, and *Marinomonas* spp., seem to branch some distance from the other γ-Proteobacteria and are related to halotolerant bacteria. The genera *Sphingomonas* and *Rhizomonas* have been identified as nonphotosynthetic members of the α-4 subclass of the Proteobacteria. Their close phylogenetic relationship is also indicated in our phylogenetic tree, where they cluster together. The genus *Sphingomonas* (previously assigned to the *Pseudomonas paucimobilis* group) is known for its ability to degrade aromatic compounds of environmental concern (34). A *Sphingomonas* strain has earlier been shown to be a dominant member of the bacterial community in Resurrection Bay in Alaska (44). Further analysis indicated that this strain had a high-affinity nutrient uptake system in nutrient-limiting conditions and was remarkably resistant to nutrient starvation and temperature stress (9). The detection of the nodule-forming *Rhizobium loti* (SCB25; similarity value, 0.97) found 25 km offshore Scripps Pier was surprising (Fig. 2). The biogeochemical implications of this finding will depend on further analysis of the presence of the *nif* gene cluster in this isolate (24).

We have shown that marine bacteria with the ability to form colonies on solid media occupy a considerably larger fraction of the marine bacterial community than the number of CFU would suggest. This is a critical finding for the future characterization of aquatic microbial communities. It suggests that

the marine isolates stored in culture collections deserve to be characterized. Exploring this biodiversity is an attractive task since these organisms possibly host a great potential for diverse applications (14). Finally, the moderate diversity of the bacterioplankton community found in this study might encourage the search for factors determining population dynamics and succession of species, ultimately affecting our understanding of marine biogeochemical processes.

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