Succession of Pelagic Marine Bacteria during Enrichment: a Close Look at Cultivation-Induced Shifts

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Enrichment experiments with North Sea bacterioplankton were performed to test if rapid incubation-induced changes in community structure explain the frequent isolation of members of a few particular bacterial lineages or if readily cultivable bacteria are common in the plankton but in a state of dormancy. A metabolic inhibitor of cell division (nalidixic acid [NA]) was added to substrate-amended (S+) and unamended (S−) grazer-free seawater samples, and shifts in community composition and per cell DNA and protein content were compared with untreated controls. In addition, starvation survival experiments were performed on selected isolates. Incubations resulted in rapid community shifts towards typical cultivable genera rather than in the activation of either dormant cells or the original DNA-rich bacterial fraction. Vibrio spp. and members of the Alteromonas/Cowella cluster (A/C) were selectively enriched in S+ and S−, respectively, and this trend was even magnified by the addition of NA. These increases corresponded with the rise of cell populations with distinctively different but generally higher protein and DNA content in the various treatments. Uncultured dominant γ-proteobacteria affiliating with the SAR86 cluster and members of the cultivable genus Oceanospirillum were not enriched or activated, but there was no indication of substrate-induced cell death, either. Strains of Vibrio and A/C maintained high ribosome levels in pure cultures during extended periods of starvation, whereas Oceanospirillum spp. did not. The life strategy of rapidly enriched cultivable γ-proteobacteria could thus be described as a “feast and famine” existence involving different activation levels of substrate concentration.

Our knowledge about the phylogenetic lineages that contribute to the marine bacterioplankton is presently obtained from three sources: isolation of various bacterial strains (33, 43), clone libraries of 16S ribosomal DNA (rDNA) (31, 37, 43), and hybridizations to whole cells or isolated nucleic acids (22, 33, 38). The results of isolation and of clone libraries often disagree. During the last decade the discrepancy between isolation and cloning has commonly been regarded as an indication of cultivation-induced shifts (4). Yet, since cloning does not reveal community structure either, this view is actually based on little experimental evidence. On the contrary, by using quantitative genome probe hybridizations against community DNA, some isolates (Sphingomonas and Caulobacter spp.) have been shown to represent a significant amount of the total bacterioplankton in brackish Baltic Sea waters (33). A marine isolate related to Vibrio was described to exhibit remarkable annual variation in population density, ranging from undetectably low to ≥100 of total community DNA (38). Is this high relative abundance of typical cultivable bacteria the exception or the rule? In a recent study on North Sea bacterioplankton (14), we found that the most readily cultivable bacteria on media low in organic carbon, such as Vibrio, Alteromonas, and Pseudoalteromonas, did not significantly contribute to the bacterioplankton community during different seasons, as determined by fluorescence in situ hybridization (FISH) with specific oligonucleotide probes. In contrast, a FISH probe targeted to 16S rDNA clones affiliating with a cosmopolitan γ-proteobacterial lineage, SAR86 (1, 14, 18, 31), detected a prominent fraction (up to 10%) of the microbial community in situ. However, no corresponding isolates were obtained in spite of extensive cultivation efforts.

It has, however, been claimed that a supposedly typical marine isolate was undetectable in situ by FISH because of its low per cell ribosome content (40). This raises the question of whether the readily cultivable bacteria of our previous study were really rare in situ, or whether they were simply not detectable by fluorescent probes. If FISH sensitivity limits are interfering with the in situ quantification of such cells, their “activation” should be observable during enrichment on substrates successfully used for their cultivation. If frequently isolated bacteria are, however, found to be rare in situ, they should then be able to take advantage of cultivation-associated changes in their environment more rapidly than their competitors. Monitoring dilutions of North Sea bacterioplankton with seawater that is free of bacteria by flow cytometry and subsequent FISH of sorted cells have provided first evidence that members of the γ-subclass of the Proteobacteria may indeed be selectively enriched (17), but it is unknown if those γ-proteobacteria were affiliated with typical marine isolates. In this context, the other side of the observed phylogenetic differences between marine isolates and rDNA clones needs to be addressed, too: how do so-called “unculturable” bacteria develop during the early phases of cultivation attempts or during typical cultivation-associated procedures, such as filtration, confinement, substrate addition, temperature variation, etc.?

We set up enrichments with substrate-amended (S+) and unamended (S−) North Sea water and subsequently analyzed community composition and changes in bacterial per cell DNA and protein content by FISH and flow cytometry. The antibiotic nalidixic acid (NA) (27) was added to half of the treatments. It inhibits prokaryotic DNA replication, yet allows cells to increase in volume. In our study, NA was not applied for the quantification of active bacteria. We rather wanted to test if
readily culturable bacteria are frequent but inactive or dormant, and if consequently their low per cell ribosome content could be the reason why we found low in situ abundances of such genera by FISH in a previous study (14). In addition, the FISH detectability of different γ-proteobacterial isolates during starvation was monitored.

MATERIALS AND METHODS

Sampling site and fixation. In August 1998, surface water was collected at a 1-m depth in acid-washed, seawater-preincubated 50-liter polyethylene containers at station Helgoland Roads (54°09′ N, 7°52′ E) near the island of Helgoland, which is situated approximately 50 km offshore in the German Bay of the North Sea. Water was stored at 4°C and further processed within approximately 1 h. Samples for flow cytometry were fixed with formaldehyde (final concentration, 2% [wt/vol]) and stored frozen. For FISH, portions of 10 to 100 ml of unfertilized seawater were fixed with formaldehyde (final concentration, 2% [wt/vol]) for several hours, collected on white polycarbonate filters (diameter, 47 mm; pore size, 0.2 μm; type GTTP; Millipore, Eschborn, Germany), and rinsed with distilled water. Filters were stored at −20°C until further processing.

Total cell counts and protein and DNA content per cell. Determination of total cell numbers and relative DNA and protein content of bacteria after double starvation was performed by flow cytometry on a FACStar Plus flow cytometer as described (Becton Dickinson, Mountain View, Calif.) (48). At least 2,000 Hocchtst 33422-positive cells were counted per sample.

Growth experiments. For the experimental enrichments, seawater was gently filtered through cellulose nitrate filters (diameter, 47 mm; pore size, 1.2 μm; Sartorius AG, Göttingen, Germany). Half of the prefiltred samples were supplemented with NA (30 mg/liter) (27). Triplicate 150-ml aliquots were incubated at the in situ temperature (16°C) on a rotation shaker (100 rpm) either unamended with NA (30 mg/liter) (27). Triplicate 150-ml aliquots were incubated with either unamended (S−) or amended (S+) treatments (estimated generation time, 83 h). Incubation with NA resulted in a concomitant increase in cell numbers, but significant in the S−−NA− and S−NA+ throughout the experiment. FISH detection in the substrate-amended treatments increased from 75% ± 1.5% to 87% ± 1.5% of total cells at the end of the incubations.

The amount of cells hybridizing with the group-specific probe for the γ-subclass of the Proteobacteria, GAM42a, increased from 2.1(±0.4) × 10^6 (n = 12) cells per ml to 4- to 5-fold and 9- to 10-fold in S− and S+, respectively. This effect was enhanced by incubation with NA. Thirteen percent ± 3% (n = 6) of total cells hybridized with probe GAM42a in the beginning and 40% (34 to 44%, S−NA−) and 72% (67 to 76%, S+NA+) after 43 h of incubation. Members of the SAR86 cluster, which are small rods (approximately 0.5 μm in width and 1 μm in length), were detected by FISH with probe SAR86-1249. They showed only weak FISH signals and could not be enriched during different treatments (Fig. 2). Their absolute cell numbers remained constant in both S−NA− and S+NA− treatments (estimated generation time, 83 h). Incubation with NA resulted in a continuous decrease in SAR86 cell numbers within 43 h of incubation. The relative abundances of this phylogenetic group dropped from 11.3% (9.0 to 14.1%) to 6.7% (5.2 to 9.1%) or below the detection level (<1% DAPI) in the various treatments.

Bacteria targeted by the oligonucleotide probe OCE232, specific for the genus Oceanospirillum (Table 1), showed only weak fluorescence and were initially present in small numbers. During incubation, Oceanospirillum spp. were not enriched significantly in any of the treatments, and their relative abundances hardly exceeded the lower limit of FISH detection rates. The Alteromonas/Colwellia cluster (A/C), as identified by oligonucleotide probe ALTT1413, showed a different response (Fig. 2). These bacteria, usually large cells compared to other marine bacteria, showed bright FISH signals. In the beginning of the experiments, they constituted approximately 1.5% of total bacteria, but increased significantly in the S−, S+, and, during the second half of the incubation period, in both NA+ treatments. Concomitantly with a rise in numbers, the cell volume of these large cells increased even more (Fig. 3). A/C constituted 6% and 20 ± 2% of total bacteria in S− after 20 and 43 h, respectively. From abundance changes, we estimated a generation time (g) of 9 h. This enrichment was enhanced by the presence of NA. The relative abundance of A/C was 33% ± 5% in the S−NA− treatment at the end of the experiment. After a strong initial increase in absolute numbers in S+NA−, numbers stagnated after 20 h of incubation, resulting in 10% ± 0.2% relative abundance (g = 11.8 h). S+NA+ treatments resulted in little increase in A/C in absolute numbers.

### TABLE 1. Oligonucleotide probes for in situ hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Probe sequence (5′→3′)</th>
<th>Target size* (16S rRNA positions)</th>
<th>% Formamide in buffer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALTT1413</td>
<td>A/C</td>
<td>TTTCGATCCCACTCCCAT</td>
<td>1413–1430</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>SAR86-1249</td>
<td>SAR86 cluster</td>
<td>GGCCTAGGGTCGTTCTTG</td>
<td>1240–1265</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>V G</td>
<td>Vibrio</td>
<td>AGGCCACAGCTCCAAGTAT</td>
<td>841–822</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>OCE232</td>
<td>Oceanospirillum</td>
<td>AGTTAATCTCATGAGCCG</td>
<td>232–249</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>G Rb</td>
<td>Rhodobacter/Roseobacter</td>
<td>GTCGATCGAGCCGATGAG</td>
<td>645–626</td>
<td>30</td>
<td>20</td>
</tr>
</tbody>
</table>

* Escherichia coli numbering (9).
group during incubation, but only in the S+N+ treatments (Fig. 2). The increase in cell numbers was even stronger in the S+N- treatment. At the end of the experiment Vibrio spp. constituted 25% ± 1% (g = 6.3 h) and 65% ± 1% of total bacteria in the S+N- and S+N+ treatments, respectively.

The two other studied groups, α-proteobacteria and Cytophaga/Flavobacterium (C/F), which constituted 23% (17 to 27%) and 31% (26 to 34%) of total bacteria in the beginning of the experiment, respectively, exhibited much lower growth during the enrichments. In absolute numbers, members of the C/F cluster almost doubled from 4.9(±0.9) × 10^5 (n = 8) to 10.4(±1.5) × 10^5 (n = 4) cells per ml in both S- and S+, whereas α-proteobacteria only grew in the S+ treatments. Both groups decreased little in their relative abundances during incubations without NA. In contrast, α-proteobacteria constituted less than half and members of the C/F cluster about one third of their original relative abundances in the S+N+ treatment.

The morphologically diverse Rhodobacter/Roseobacter sub-group of the α-subclass of the Proteobacteria constituted 9% ± 3% of total bacteria and a significant fraction (40%) of α-proteobacteria (Table 1). Mean cell numbers of Rhodobacter/Roseobacter increased from 1.5(±0.5) × 10^5 (n = 8) by 1.7-fold and by 2.2-fold in the first 20 h of incubation in the S- and S+ treatments, respectively, whereas the increase in NA treatments was smaller. Within the second half of incubation, numbers of cells targeted by probe G Rb changed little in all treatments and dropped below the original value in the substrate-amended treatments (Fig. 2). Their relative abundances in all but the NA- treatments decreased during 43 h of incubation. Rhodobacter/Roseobacter constituted only about 4% ± 1% in S+ but up to 7% ± 1% of bacteria in the S- treatments.

Changes in per cell DNA and protein content. The flow cytometric signature of double-stained bacterioplankton cells revealed treatment-specific changes during the incubations (Fig. 3). At the end of the experiment, the cytograms from the unamended treatments with and without NA showed pronounced differences from those of the original community. In both, a second cell population with higher protein content was discernible after 43 h. Incubation with substrates either with or without NA resulted in the appearance of cells with significantly higher DNA and protein content than in the unamended sample. In S+N+ treatments, the fraction of these large cells was much higher (56.0%) than in S+N- (19.8%).

Starvation experiment. Representative isolates obtained from the North Sea (14) hybridizing with probe ALT1413, OCE232, or G V were starved for more than 50 days (Fig. 4). The percentage of intact bacteria was determined as the fraction of ribosome-containing cells, i.e., by their EUB338 signal. A/C and Vibrio spp., which were readily enriched during the field growth experiment, showed no significant loss of EUB338 detection rate versus DAPI during 50 days. In contrast, the detection rates of Oceanospirillum spp. decreased rapidly within the first 20 days, and cells from this strain were almost not detectable in the last half of the starvation experiment. The addition of fresh medium did not result in an increase in FISH-detectable cell numbers of Oceanospirillum within 10 h of incubation.

**DISCUSSION**

Shifts in bacterioplankton community composition during enrichment. Enrichment cultures have a long tradition in microbiology (8). This experimental strategy, arranged intentionally or not, eventually resulted in the isolation of the presently known variety of marine bacteria. The classic ZoBell approach...
of 1946 (33, 47) has been repeatedly improved or modified, e.g., by differential filtration (15), dilution (10), and the use of specific substrates (24). Knowledge about the spatiotemporal occurrence or physiological features of particular phylogenetic groups may allow the design of more directed experiments (12, 23, 36). However, enrichment attempts always represent substantial interferences with microbial life and their environment, even in the absence of additional substrates. For example, prefiltration may influence bacterioplankton composition by removal of large filamentous and most of the particle-attached cells (1, 13). Cellulose ester filters of 1.2-mm pore size were found to retain up to almost 50% of unfiltered bacterial abundances in coastal waters (19). We could, however, not verify such a reduction in our samples (means ± standard deviation: unfiltered, 1.52 ± 0.04 × 10^6 cells ml^{-1}, n = 3; prefiltered, 1.57 ± 0.19 × 10^6 cells ml^{-1}, n = 9). Even gentle filtration may increase substrate concentrations (e.g., of dissolved free amino acids) due to damage of phytoplankton cells (15) and disrupt the link between dissolved and particulate organic matter (34). The absence of protistan grazers will relieve bacteria from selective mortality (41), and confinement will put an end to the dynamic equilibrium between the formation and decomposition of organic matter (45).

Already in 1984, Ammerman et al. (5) and Ferguson et al. (15) had shown an increase in population size and average cell volume during undirected bacterioplankton growth in unamended seawater. More recently, FISH in combination with flow cytometry revealed changes in the taxonomic community...
composition of North Sea bacterioplankton in dilution culture (17). In our experiments, changes in community structure occurred more rapidly than reported by Suzuki, who did not detect taxonomic shifts in filtered seawater samples for a period of 24 h (42). \(\gamma\)-Proteobacteria had increased overproportionally already after 20 h of incubation in S\(^-\), whereas \(\alpha\)-proteobacteria and C/F members did not (Fig. 1). The addition of organic substrates in micromolar concentrations (5.7 mg of C per liter) did not result in significantly higher total cell numbers after 48 h compared to S\(^-\) (Fig. 1), but in an even more pronounced change in community structure. \(\gamma\)-Proteobacteria increased from about 15 to 60% and the fraction of C/F again remained constant, but \(\alpha\)-proteobacteria decreased by half in relative abundance. Concomitantly, the development of cell populations with higher protein and DNA content was observed in S\(^+\) and S\(^-\) (Fig. 3), and these large cells thus mainly belonged to the rapidly growing fraction within the \(\gamma\)-proteobacteria.

Our study extends previous findings in several respects. We present the response of several individual groups within the marine \(\gamma\)-proteobacteria to different treatments. Evidence is provided that the dominant members of this lineage in situ were rapidly outcompeted during enrichment culture. In the prefiltered seawater, about 14% of total cell numbers, corresponding to approximately \(2 \times 10^7\) cells ml\(^{-1}\), belonged to the \(\gamma\)-subclass of Proteobacteria. Members of a single phylogenetic lineage, the uncultured SAR86 cluster, formed 90% of all \(\gamma\)-proteobacteria in the beginning of the experiments (Fig. 5). SAR86 belongs to the free-living fraction of the pelagic bacterioplankton, as determined by clone libraries of prefiltered seawater (1, 14) or visualization by FISH (14). Several typical culturable \(\gamma\)-proteobacterial genera were detected in very low numbers, either attached (\textit{Vibrio} and \textit{Alteromonas}) or free-living (\textit{Oceanospirillum}) in the original North Sea pelagic community (14). In contrast to \textit{Vibrio} and A/C, SAR86 and \textit{Oceanospirillum} were not enriched in any of the treatments; the absolute abundances of these groups remained constant, and we did not observe a significant increase in either cell size or FISH signal intensity. Members of the SAR86 cluster and \textit{Oceanospirillum} were therefore neither visibly subjected to substrate-accelerated death (35) nor activated in either S\(^-\) or S\(^+\). Enhanced mortality of SAR86 was, however, observed as a consequence of the antibiotic treatment, and already within the first 20 h of incubation, the abundance of SAR86 had decreased significantly in the NA\(+\) incubations (Fig. 2). NA might, therefore, have acted as a cell toxin for members of this lineage (2) or represented a stress factor that caused the lysis of virus-infected cells (46).

On the other hand, \textit{Vibrio} and A/C responded rapidly to our simulated culturing conditions, with lag phases ranging from 5 to 10 h and generation times of between 7 and 12 h, as estimated from the abundance changes during the 43-h experimental period. This corresponds well with the high numbers of \textit{Vibrio}-related sequences found in clone libraries of stationary-phase dilution cultures from Mediterranean Sea samples (20), and with the selective enrichment of bacteria affiliating with \textit{Alteromonas macleodii} during enclosure incubations in the same system (39). Presently we cannot distinguish between the different potential causes for the observed community shifts, such as shorter response times to substrate upshifts, but also antibacterial or autocrine growth factors released by the rapidly growing groups (44).

No activation of dormant culturable bacteria. The high relative contribution of microbes affiliated with \textit{Vibrio}, \textit{Alteromonas}, or \textit{Colwellia} to the colony-forming bacteria (14, 28) might be attributed to rapid cell multiplication and short lag times. Alternatively, it may be the consequence of a high fraction of dormant cells from these genera in the original community that are activated by culturing effects like substrate addition or the presence of solid surfaces (26). The combined incubation with NA and substrates causes an abnormal increase in cell size, and consequently ribosome content (11), by delaying cell division until the eventual appearance of NA-resistant strains (Fig. 3). Therefore, dormant bacteria that respond to substrate addition should become FISH detectable in such a treatment. In our experiments, the offered substrate mix was appropriate to activate, e.g., \textit{Vibrio}, \textit{Alteromonas}, and \textit{Colwellia}, as it has been successfully utilized for their isolation previously (14). However, no increase in the relative abundances of these typical culturable bacteria was observed during the initial period of incubation with NA (0 to 20 h) (Fig. 1 and 2). In contrast, there was a clear rise of the two groups during the same incubation period in the treatments lacking NA. This is evidence that no or few dormant, FISH-undetectable bacteria affiliated with \textit{Vibrio} or A/C were present in the water column. In fact, no initial increase in total FISH detection rates with the bacterial probe EUB338 was observed during incubations with NA. This suggests either that in general there was no activation of dormant cells by our incubation conditions or else that no cells escaped FISH detectability due to their low ribosome content.

Interestingly, the addition of the cell division-inhibiting agent NA did not result in the dominance of one particular resistant bacterial group irrespective of substrate levels, but rather amplified the success of the most competitive lineage within the respective treatment. In the substrate-unamended enrichments, resistant strains of the A/C cluster increased to similar absolute numbers in NA\(^-\) as in NA\(+\) after 43 h of incubation. However, in the presence of the antibiotic, they constituted a much larger fraction, about one third of the total community. The addition of substrate always specifically favored \textit{Vibrio}. This group constituted 65% of total bacteria in NA\(+\), which was almost three times as much as in the NA\(-\) treatment (Fig. 5). On the other hand, A/C, in spite of being potentially NA resistant (Fig. 5), was almost completely suppressed in substrate-amended NA\(+\) treatments, and the anti-
biotic shifted the competition between the two groups towards *Vibrio*. It would be premature to draw general conclusions from an unplanned observation in a single sample. However, the study of the combined effects of growth-promoting and growth-inhibiting factors on microbial competition might be a fruitful field for future investigations.

**Enrichable culturable genera: “feast-or-famine” strategists.** Our data do not support the hypothesis that readily culturable pelagic bacteria are in general rapidly enriched in filtered or substrate-amended seawater. During extensive cultivation at Hegoland Roads (14), 33 of 145 different bacterioplankton isolates affiliated with genera which also dominated our enrichments. However, another nine isolates were related to *Oceanospirillum*, which did not grow during the incubations. Strains related to *Vibrio* and A/C maintained large amounts of cellular ribosomes during starvation in pure culture, whereas the FISH detectability of *Oceanospirillum* declined rapidly (Fig. 4). A high total per cell rRNA content of nongrowing cells apparently provides the potential for a more rapid response to changes in growth conditions (16). We conclude that rapidly enriched culturable bacteria like *Vibrio* and A/C are able to maintain a high potential to react to changes in growth conditions even during extended periods of nongrowth. This life strategy goes beyond the simplified dichotomy of r versus K selection (6), and the growth of the two r strategists A/C and *Vibrio* was apparently triggered at different ambient substrate concentrations (Fig. 5). Members of both lineages have been found associated with marine metazoans (7, 25), which would agree with a concept of a feast-or-famine existence.

This bacterial life strategy will confront microbiologists trying to culture as yet uncultured bacteria with fundamental problems. Some representatives (e.g., A/C) grow on unamended seawater and media with a relatively low carbon content (14). On the other hand, they maintain a high potential for growth during starvation and show immediate response to the environmental changes caused by sampling. Moreover, members of several readily culturable genera survived and rapidly resisted the stress factor NA. In summary, new strategies are required to enrich and eventually isolate yet uncultured bacteria in plankton samples, and for this purpose molecular methods that monitor the changes in community composition will be essential.

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