Pelagische Bakteriengesellschaften in der Nordsee: Kultivierung, Diversität und *in situ* Dynamik

Pelagic Microbial Communities in the North Sea: Cultivation, Diversity, and *In Situ* Dynamics

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Heike Eilers geb. Stürmeyer

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1. Gutachter: Priv.-Doz. Dr. Rudolf Amann
2. Gutachter: Prof. Dr. Ulrich Fischer

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INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Im Rahmen dieser Arbeit wurde die mikrobielle Lebensgemeinschaft des Pelagials der Nordsee (Station Kabeltonne vor Helgoland) untersucht. Bakterien wurden kultiviert und Methoden zur Isolierung bisher nicht kultivierter Bakterien entwickelt, um erste Erkenntnisse über die Diversität und Lebensstrategien mariner Bakterien zu gewinnen. Des weiteren wurde eine 16S rDNA Genbank aus Umwelt-DNA erstellt. Nach vergleichender phylogenetischer Analyse von 16S rDNA Sequenzen der Isolate und Klone wurden spezifische Oligonukleotidsonden entwickelt. Abundanzen und Sukzessionen der häufigen phylogenetischen Gruppen wurden mit Hilfe der Fluoreszenz-*in-situ*-Hybridisierung (FISH) im Bakterioplankton innerhalb eines Jahres und in Anreicherungsexperimenten mariner Bakterien bestimmt.

Vertreter der Bacteria dominierten im Jahresverlauf 1998 die prokaryontische Lebensgemeinschaft. Diese setzte sich im Frühling und Sommer vorwiegend aus Vertretern der *Cytophaga/Flavobacterium*-Gruppe (CF) (ca. 55% aller Bakterien) und den α- und γ-Proteobakterien zusammen (je ca. 20% aller Bakterien). Während einer Diatomeensukzession im Frühling entwickelten sich zuerst CF, dann α-Proteobakterien und später γ-Proteobakterien. Im Sommer erreichten CF, α- und γ-Proteobakterien gleichzeitig ihr Jahresmaximum; dabei wurden die höchste Wassertemperatur, die niedrigste Konzentrationen von anorganischem Phosphor und Stickstoff und eine Diatomeenblüte von *Lauderia* spp. im Jahresverlauf beobachtet.

Cytophaga-Isolate, die nur einen kleinen Anteil der kultivierten Bakterien in der Stammsammlung repräsentierten, stellten im Frühling und Sommer einen Anteil von 6% aller Bakterien dar. Im Gegensatz dazu stammten häufig kultivierte Bakterien des marinen Pelagials aus der Klasse der γ-Proteobakterien. Vertreter der Gattungen *Vibrio*, *Alteromonas* und *Colwellia* waren aber *in situ* selten. Als partikelassoziierte, copiotrophe Bakterien sind sie kurzfristig metabolisch sehr aktiv. Nach überstandenen Hungerperioden, verfügen sie mit einer hohen Ribosomenanzahl über eine Anpassung, die ein schnelles Wachstum bei Verfügbarkeit hoher Nährstoffkonzentrationen ermöglicht. Innerhalb der Gruppe copiotropher Bakterien entscheidet die Substratkonzentration, ob ausschließlich ein Wachstum von *Alteromonas* spp. und *Colwellia* spp. erfolgt oder bei höheren Konzentrationen *Vibrio* spp. dominiert. In Anreicherungskulturen und Plattierungen von marinen Bakterien wurde die Ursprungspopulation sukzessiv von diesen Bakterien überwachsen.

Bisher nicht kultivierte, kosmopolitische Gruppen der γ-Proteobakterien wurden mit Hilfe der 16S rDNA Genbank detektiert. Vertreter der NOR5 (NOR=Nordsee) und SAR86 (SAR=Sargasso See, Atlantik) Gruppen wurden erstmals *in situ* mittels spezifischer 16S rRNA-gerichteter Oligonukleotidsonden visualisiert. Es handelte sich im Gegensatz zu vielen häufig kultivierten Bakterien um freilebende Bakterien, die mit bis zu 10% aller Bakterien *in situ* dominierten.

In dieser Arbeit konnte erstmals ein Stamm der NOR5 Gruppe durch die Modifikation der Kultivierungsmethode isoliert werden. Erste Versuchsergebnisse sprechen dafür, daß es sich beim Stamm KT71 um ein langsam wachsendes, oligocarbophiles Bakterium handelt. Der Stamm KT71 repräsentierte *in* situ von Juni bis August 1998 im Durchschnitt ca. 5% des Bakterioplanktons. Das Wachstum zu Zelldichten von bis zu $1{,}3\times10^5$ pro ml war verknüpft mit niedrigen Phosphor- und Stickstoffkonzentrationen, dem Aufkommen der Diatomeen *Lauderia* spp. und moderaten Wassertemperaturen.

SUMMARY

In this thesis, the microbial community of the North Sea bacterioplankton at Helgoland Roads was studied. Diversity and life strategies of marine bacteria were investigated by cultivation experiments and methods developed for the isolation of previously uncultured strains. Additionally, a 16S rDNA clone library was established. Specific oligonucleotide probes were designed after comparative sequence analysis of bacterial 16S rDNA sequences from isolates and clones. Abundances and succession of the phylogenetic groups were monitored during a season at station Helgoland Roads and during an enrichment experiment by fluorescence *in situ* hybridization (FISH).

During 1998, members of the domain Bacteria dominated the prokaryotic community. The *Cytophaga/Flavobacterium* group (CF) (55% of all bacteria) and α- and γ-proteobacteria (each 20% of all bacteria) dominated microbial community in spring and summer. During a succession of diatom blooms in the spring first CF, then α-proteobacteria, and finally γ-proteobacteria developed. In summer, on the other hand, the tree groups developed at the same time. This coincided in 1998 with highest water temperature, lowest concentrations of inorganic phosphorous and nitrogen, and a bloom of diatoms (*Lauderia* spp.).

Cytophaga isolates were rare in the culture collection, but they constituted 6% of all bacteria during spring and summer by FISH. In contrast, γ-proteobacteria were readily culturable from the pelagic zone; but *Vibrio* spp., *Alteromonas* spp. and *Colwellia* spp. were rare *in situ* . These particle-associated copiotroph bacteria are metabolically very active. Even after starvation these cells contain excess ribosomes, which we hypothesise allow them to grow rapidly when nutrients become available. By enrichment cultures and plating experiments, we found that at lower substrate concentrations only *Alteromonas* spp. and *Colwellia* spp. grow, while at higher concentrations *Vibrio* spp. dominate. The original mixed population was overgrown by these bacteria.

As yet uncultured, cosmopolitan groups of γ-proteobacteria were detected by constructing a 16S rDNA clone library. Members of the NOR5 (NOR=North Sea) and SAR86 (SAR=Sargasso Sea) groups were visualized by specific oligonucleotide probes targeting 16S rRNA. In contrast to the rapidly cultivated bacteria discussed above, they are freeliving. They constitute up to 10% of all bacteria *in situ*.

During this work, a strain of the NOR5 group was isolated by modification of cultivation strategy. Our growth experiments suggest that strain KT71 is an oligocarbophilic marine bacterium. It constituted on average 5% of bacterioplankton during June and August 1998. We hypothesisze that the combination of low phosphorous and nitrogen concentrations, a bloom of *Lauderia* spp., and moderate water temperatures led to high *in situ* cell densities of as much as $1{,}3\times10^5$ per ml.

Teil I

Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

Mikroorganismen spielen im Ökosystem Meer eine bedeutende Rolle für die Stoffkreisläufe der Biosphäre. Verschiedenste Biotope, wie Küsten- und Tiefseesedimente, Felsen, Oberflächen von Pflanzen und Tieren, absinkende Partikel und das Freiwasser enthalten Biozönosen, die in ihren Artenzusammensetzungen, deren relativen Anteilen und in ihrer biogeochemischen Funktion einzigartig sind. Die Isolierung von Mikroorganismen und Kultivierung in Reinkulturen ist unverzichtbar, um ihr physiologisches Potential experimentell zu erforschen. Das Verständnis ihrer komplexen ökologischen Interaktionen geht einher mit ihrer Identifizierung und der Bestimmung von Verbreitung und Häufigkeit dieser Mikroorganismen in ihrem Lebensraum.

1. Die marine Bakterioplanktongemeinschaft

Die Größe und Komplexität des Ökosystems Meer machen es schwierig, es als Einheit zu untersuchen. Deshalb wird es in seiner Verikalstruktur in Benthal und Pelagial unterteilt. Im freien Wasser des Pelagials hält sich das Plankton auf. Dazu gehören das Phytoplankton, das Zooplankton und schließlich das Bakterioplankton.

Das Bakterioplankton wird im allgemeinen als Schlüssel des biogeochemischen Haushalts mariner Ökosysteme betrachtet. Bis heute weiß man wenig über die Arten, die diese Gemeinschaft ausmachen, denn die geringe Größe dieser Organismen erschwert das Studium ihrer Diversität und Ökologie (Abbildung 1). Anders als in der Botanik und Zoologie reichen morphologische Merkmale nicht bzw. nur sehr begrenzt aus, um Prokaryonten des Pikoplanktons [154] zu identifizieren.

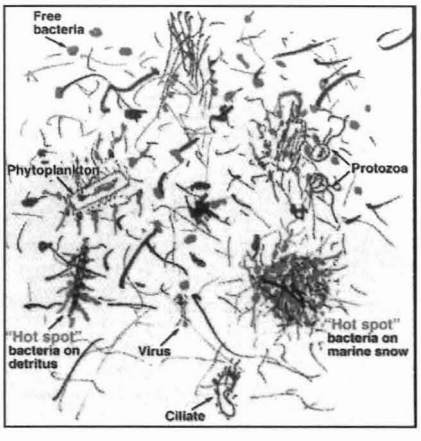


Abbildung 1. „Microbial loop". Impressionistische Version [10].

2. Heterotrophe Bakterien im Nahrungsnetz

Die ineinandergreifenden Muster von Fraß- und Detritusnahrungsketten werden als Nahrungsnetz bezeichnet. Als Primärproduzenten stehen im Meer hauptsächlich einzellige Algen und Cyanobakterien, die als Phytoplankton zusammengefaßt werden. Die Nahrungskette („grazing food chain") setzt sich im Zooplankton über mehrere trophische Ebenen fort und endet mit den Fischen als Endkonsumenten. In diese Kette fließt ein großer Teil der Primärproduktion ein [27]. Andererseits setzten Phytoplankter einen Teil des assimilierten Kohlenstoffs als gelöstes organisches Material frei [15]. Heterotrophe Bakterien nehmen das gelöste organische Material auf und bauen Reste abgestorbener Phyto- und Zooplankter hydrolytisch ab [80]. Dabei erfolgt nicht nur eine Exkretion gelöster anorganischer Nährstoffe im Zuge der Mineralisation (Abbildung 2), sondern auch eine bakterielle Sekunkärproduktion. Heterotrophe Bakterien stellen aufgrund ihrer Produktivität den größten Anteil an der Biomasse von Mikroorganismen des Epipelagials [55]. Somit ist der hohe Stellenwert der heterotrophen Bakterien des marinen Pelagials ist unbestritten, zumal sie 50% des Kohlenstoffs, der durch Photosynthese fixiert wurde, konsumieren [27] und gleichzeitig 50% des partikulären organischen Materials darstellen [25]. Im mikrobiellen Nahrungsnetz („microbial loop") dienen sie außerdem selbst dem Mikro- und Nanoplankton, das sind Ciliaten, mixo- und heterotrophe Flagellaten, als Substrat [11]. Dieser Fraß durch Protisten [53, 165, 181] und die Infektion mit Bakteriophagen [47, 131] hat einen hohen Einfluß auf die biogeochemischen Stoffkreisläufe.

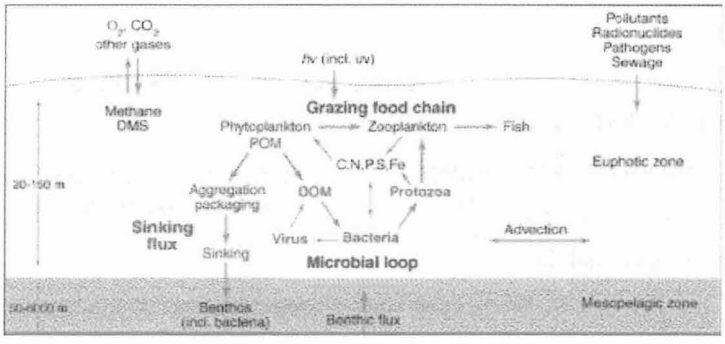


Abbildung 2. Pelagisches marines Nahrungsnetz. Klassische Version [10].

Um die Ökologie des Bakterioplanktons zu verstehen, wurden Prozesse, wie Sekundärproduktion [48, 157], Wachstum [48, 171] und andere Aktivitäten [12, 32], sowie

die Gesamtkonzentrationen von z. B. Mikroorganismen, deren Biomassen und Bakteriochlorophyll [27, 182] in biogeochemischen Studien bestimmt. Darin wurde die enge Beziehung zwischen Primärproduzenten, Bakterien und Nährstoffen aufgezeigt. In gemäßigten Zonen unterliegt das Phytoplankton einer saisonalen Sukzession. Damit einhergehend ändert sich die Gesamtzellzahl und Zusammensetzung des Bakterioplanktons. Dies wurde erstmals durch DNA-DNA-Hybridisierungen zu verschiedenen Jahreszeiten demonstriert [99]. Murray et al. zeigten, daß Archaea im Winter einen höheren Anteil am rRNA-Gehalt der mikrobiellen Gemeinschaft darstellen, während Gruppen der Bacteria im Sommer abundant sind [69].

3. Identifizierung heterotropher Bakterien

Heterotrophe Bakterien wurden in einer „black box" zusammengefaßt, weil viele Bakterien nicht identifiziert waren. Dies führte lange zum Verlust von Einblicken in ökologische Interaktionen wie z.B. Konkurrenz unter Prokaryonten, Einfluß der viralen Infektion und Fraß durch protistische Räuber. Wer sind die heterotrophen Bakterien, das entscheidende Glied im „microbial loop"?

In den Anfängen der Mikrobiologie konzentrierte sich die Bakterienidentifizierung im Wesentlichen auf die Morphologie, (Abbildung 3), auf cytologische Merkmale und auf Stoffwechseleigenschaften.

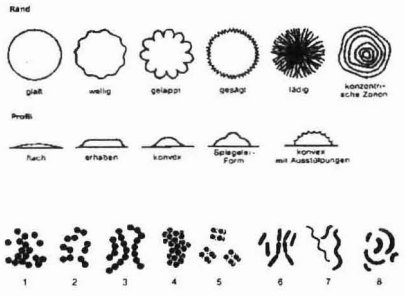


Abbildung 3. Kolonien auf Agarmedium **(A)** und Formen einzelliger Bakterien **(B)** [146, 164].

Die Molekularbiologie bietet seit Mitte der 80er Jahre weitere Methoden zur Identifizierung von Mikroorganismen. Die Entdeckung der ribosomalen RNA als hochkonservierten Merkmalsträger und ihr Sequenzvergleich ermöglichte erstmals die Erarbeitung eines phylogenetischen Stammbaums zur Rekonstruktion der Evolution [103, 176, 183]. Daraus wird eine große Diversität der Mikroorganismen ersichtlich. Mikroorganismen finden sich nicht nur in den beiden Domänen

der Prokaryonten, Bacteria und Archaea, sondern auch in der Domäne der höheren Tiere und Pflanzen, Eucarya, wieder (Abbildung 4).

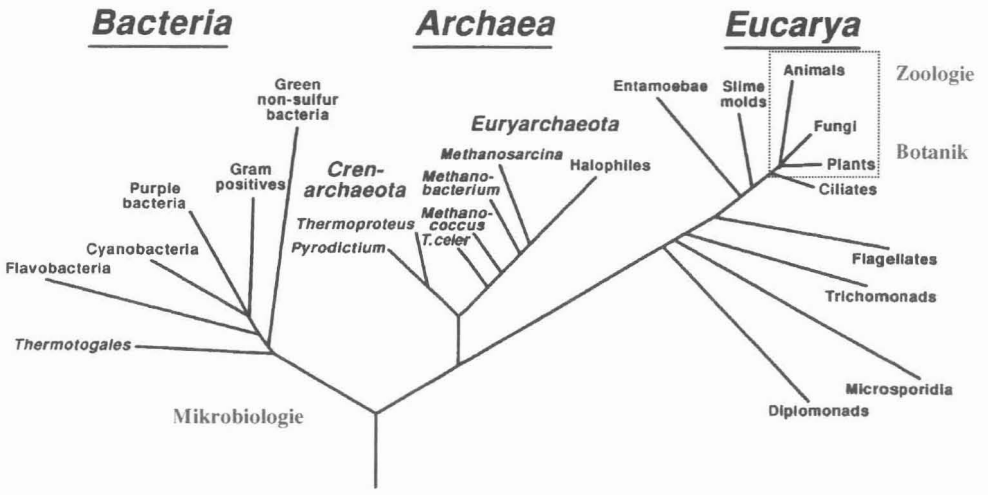


Abbildung 4. Allgemeiner phylogenetischer Stammbaum mit den drei Domänen, Bacteria, Archaea und Eucarya ([177], modifiziert).

3.1. Isolierung und Kultivierung heterotropher Bakterien

Erste Versuche, heterotrophe marine Bakterien zu identifizieren und zu quantifizieren, unternahm ZoBell in den 40ern des 20. Jahrhunderts [180]. Aus der Häufigkeit der Isolierung mariner Mikroorganismen leitete er ab, daß Vertreter der Gattungen *Pseudomonas*, *Vibrio*, *Spirillum*, *Flavobacterium*, *Achromobacter* und *Bacterium* die mikrobielle Gemeinschaft des Pelagials dominierten. [179]. Spätere mikrobiologische Arbeiten bauten auf Isolierungs- und Identifizierungsstrategien von ZoBell auf, erweiterten das bekannte Spektrum aber nur um wenige neue Gattungen [13]. Der klassische ZoBell-Ansatz findet bis heute seine Anwendung [20, 67, 99, 128, 166]. Allerdings sind auch Verbesserungen und Weiterentwicklungen der Kultivierungstechniken erfolgt. So wurden Verdünnungsreihen [23, 149] und substrat- (DMSP, Lignin, Chitin) bzw. nährstoffspezifische (P, N) Anreicherungen [29, 65, 66, 68, 98, 185] durchgeführt.

Die Identifizierung mariner Isolate durch den Sequenzvergleich der 16S rRNA zeigte, daß diese hauptsächlich aus den Phyla der Proteobakterien, der Actinobakterien (ehemals Gram-positive Bakterien mit hohem GC-Gehalt der DNA) und der *Cytophaga/ Flavobacterium/Bacteriodes* Gruppe (CFB) (Tabelle 1) stammen.

Tabelle 1. Vorläufige Liste kultivierter Bakterien*) des marinen Pelagials.

Phylum Klasse	Gattung	Representativer Stamm	Accession-Nummer	Referenzen
Proteobacteria				
α-Proteobacteria	*Azospirillum*	*A. sp.* BAL31	U63951	[128]
	Rhodobacter	*R.* sp. BAL2	U63934	[128]
	Hyphomonas	*H. jannaschiana* SW47	U85839	[20, 127]
	Octadecabacter	*O. antarcticus* 307	U14583	[20]
	Paracoccus	*P. aminovorans* prliST3	Y15324	[132]
	Roseobacter	*R. algicola* X78313	U64008	[20, 67, 98, 124, 125, 127, 132, 153]
	Sagituella	*S. stellata* E-37^T DSMZ11524	U58356	[65, 67]
	Sulfitobacter	*S. pontiacus* EE-36 DSMZ11700	AF007254	[67, 124]
	Sphingomonas	*S.* sp. strain RB2256	AF148812	[20, 124, 125, 127, 128, 149]
	Erythrobacter	*E. longus* SCB26	U64003	[124, 127]
	Erythromicrobium	*E.* sp. BAL34	U63952	[128]
	Caulobacter	*C.* sp. SKA25	AF261047	[125, 127, 128]
	Bevundimonas	*B.* sp. BAL51	AF182022	[127]
	Rhizobium	*R. loti* SCB25	U64002	[68, 124, 128]
	Agrobacterium	*A.* sp. SKA42	AF261063	[125]
β-Proteobacteria	*Alcaligenes*	*A.* sp. BAL37	U63953	[128]
	Polaromonas	*P. vacuolata* 34-P^T ATCC 51984	U14585	[70, 79]
	Rhodoferax	*R.* sp. BAL47	U63961	[128]
	Zoogloea	Z. sp. BAL43	U63957	[128]
γ-Proteobacteria	*Marinomonas*	*M. vaga* SCB50	U64027	[124]
	Shewanella	*S. putrefaciens* ACAM588	U85905	[20, 127, 128]
	Marinobacter	*M. hydrocarbonoclasticus* IC184	U85866	[20, 70, 107, 124, 125, 127, 133]
	Halomonas	*H. variabilis* SW48	U85873	[20, 38, 107]
	Vibrio	*V. splandidus* KAT16	AF025329	[70, 78, 110, 124, 125, 127, 133]
	Pseudoalteromonas	*P. atlantica* MED14	AF025557	[20, 77, 102, 124, 125, 127, 128, 133]
	Colwellia	*C. psychrerythraea* ACAM 500^T	AF001375	[17, 20, 70, 127]
	Pseudomonas	*P. stutzeri* SCB24	U64001	[20, 98, 124, 133]
	Deleya	*D. marina* ATCC25374	M93354	[38]
	Psychrobacter	*P. glancincola* IC084	U85876	[20, 125, 127]
	Photobacterium	*P.* sp. SKA34	AF255623	[125]
	Acinetobacter	*A.* sp. BAL7	AF182024	[127]
	Alcanivorax	*A.* sp. SCB54	AF052599	[124]
	Xanthomonas	*X.* sp. SCB27	U64004	[124]
	Chromohalobacter	*C.* sp. SCB23	U64000	[124]
	Aeromonas	*A.* sp. BAL19	U63945	[128]
	Moraxella	*M.* sp. ANT7	AF025577	[127]
	Glaciecola	*G. punicea* IC085	U85852	[18, 20]
	Psychromonas	*P.* sp. ICO47	U85850	[20]
	Nitrosococcus	*N. oceanus* str. C-107	M96395	[72]
Actinobacteria				
	Micrococcus	*M.* sp. MB-12	U85892	[20]
	Arthrobacter	*A. agilis* IC044	U85895	[20, 127]
	Kocuria	*K.* sp. SCB58	AF182017	[127]
	Microbacterium	*M.* sp. SCB57	AF182016	[127]
	Aureobacterium	*A. liquefaciens* prliST4	Y15325	[132]

Fortsetzung Tabelle 1. Vorläufige Liste kultivierter Bakterien*) des marinen Pelagials.

Phylum	Gattung	Representativer Stamm	Accession-Nummer	Referenzen
CFB	*Flavobacterium*	*F. uliginosum* SCB41	U64018	[20, 124, 125, 128]
	Gelidibacter	*G. algenes* ACAM551	U62916	[19, 125]
	Capnocytophaga	*C. canimorus* SCB38	U64015	[124]
	Polaribacter	*P. franzmanii* 301	U14586	[20, 70, 124]
	Psychroflexus	*P. torquis* strain ACAM 623	U85881	[20]
	Flexibacter	*F. maritimus* SCB36	U64013	[124, 125, 127]
	Cytophaga	*C. marinoflava* ATCC19326^T	AF203475	[16, 68, 128] [127]
	Flectobacillus	*F.* sp. BAL49	AF182020	[127, 128]
	Microscillia	*M.* sp. SKA27	AF261049	[125]

*) Marine Stämme, deren 16S rDNA Sequenzen in einer Datenbank hinterlegt sind.

Seitdem man die Diskrepanz zwischen der Zellzahlbestimmung durch einfache direkte Lichtmikroskopie oder später auch durch Epifluoreszenzmikroskopie nach spezifischer Färbung von Bakterien [76], und der Anzahl koloniebildender Einheiten auf Agarplatten erkannt hatte, stellte man die Kultivierungspraxis in Frage [82]. Trotz der methodischen Weiterentwicklung gilt bis heute, daß mehr als 99% aller planktischen Bakterien nicht nach der Tradition ZoBells kultiviert werden können [42], was von Staley und Konopka [161] als „the great plate count anomaly" bezeichnet wurde. In diesem Zusammenhang lieferten Studien der folgenden Jahre zum Teil sehr gegensätzliche Untersuchungsergebnisse. Im Folgenden werden ausgewählte Arbeiten thematisch geordnet kurz dargestellt.

Die niedrige Kultivierungsrate mariner Bakterien läßt sich möglicherweise auf den hohen Anteil von nicht intakten Zellen des Bakterioplanktons zurückführen. Durch DNA-Färbetechniken und Elektronenmikroskopie wurde gezeigt, daß nur bis zu einem Drittel aller pelagischen Bakterien Nukleoide beinhalten [184] bzw. nur bis zur Hälfte aller Zellen intakt, d.h. mit intrazellulären Strukturen ausgestattet sind [73]. Mikroskopische Gesamtzellzahl-bestimumungen [76] erlauben keinerlei Einblicke in den metabolischen Status der Bakterien.

In Übereinstimmung mit den cytologischen Beobachtungen wurde in nur bis zu 10% aller pelagischen Bakterien respiratorische Aktivität nachgewiesen [26, 32, 57, 88, 155]. Allerdings werden nur Bakterien mit ausreichend hoher Umsatzrate detektiert, die die Bildung eines mikroskopisch sichtbaren Kristalls ermöglicht. Andererseits wurde demonstriert, daß der verwendtete Farbstoff (CTC), der ein alternativer Elektronenakzeptor in der Atmungskette ist, toxisch auf den bakteriellen Metabolismus wirkt [173].

Bereits 1978 wurde die Teilungsaktivität von Bakterien durch den Einsatz eines Antibiotikums bestimmt, das die Teilung inhibierte, aber gleichzeitig Größenwachstum erlaubt. Der Anteil der elongierten und damit potentiell lebensfähigen Zellen („frequency of

dividing cells") ist in der Regel höher als der der kolonienbildenden Einheiten [84, 93, 142]. Allerdings ist nicht bekannt, wieviele lebende, langsam wachsende Bakterien durch Substratzugabe tatsächlich aktiviert werden können. Ein großer Teil des Bakterioplanktons wurde mittels Mikroautoradiographie (41-76%) als metabolisch aktiv gezeigt [88]. Mit diesen Daten stimmen Hybridisierungen der 16S rRNA mariner Bakterien mit fluoreszenzmarkierten universellen Oligonukleotidsonden überein. 22 bis 78% [88] bzw. 42 bis 96% [63] aller DAPI-färbbaren (DAPI= 4´-6-Diamidino-2-phenylindoldihydrochlorid) Zellen ist lebend.

Unter der Annahme, daß die niedrigen Nährstoffkonzentrationen in den Ozeanen nicht für Wachstum ausreichen könnten, wurde als Überlebensstrategie mariner Bakterien das Hungern („starvation") [7, 116] oder der Schlaf- bzw. Ruhezustand („dormancy") [87] postuliert. In diesem Zustand, auch als „viable but non-culturable" bezeichnet [143], sind viele Bakterien offensichtlich nicht in der Lage, unter Laborbedingungen anzuwachsen. Es wurde spekuliert, daß die Kommunikation unter Bakterien und die Koordination des Wachstums gesamter Populationen durch die Entnahme aus dem System gestört wird [86]. Beispielsweise wurde die Sekretion eines bakteriellen Wachstumsfaktors logarithmisch wachsender Zellen als sogenannter Wiederbelebungsfaktor für schlafende Zellen beschrieben [111, 112] und könnte demnach ein wesentlicher Bestandteil sein, der in Laborkulturen fehlt. Außerdem wurde festgestellt, daß Bakterien unter Laborbedingungen einem Substratschock unterliegen können, wenn sie sich im Hungerstatus befanden [162]. Dieser Effekt ist seit 1964 in der Literatur als „substrate-accelerated death" [130] bekannt. Außerdem wurde gezeigt, daß marine Bakterien aus der γ-Subklasse der Proteobakterien antimikrobielle Faktoren ausscheiden [81], was unter Umständen das Wachstum anderer Stämme in Anreicherungen unterbinden könnte.

Mit Beginn eines jeden Kultivierungsversuches verursachte allein die Aufbewahrung der Pikoplanktonfraktion im geschlossenen Gefäß innerhalb von wenigen Stunden eine erhöhte Koloniebildungsrate von <1% auf >40% [42], was sich vermutlich auf drastische Änderungen in der Diversität, also Artenvielfalt und -Abundanzen zurückführen läßt [46, 145]. In Flüssigkulturen erfolgte immer eine Anreicherung einer einzigen phylogenetischen Gruppe, die der γ-Proteobakterien [46, 61], was sich deutlich in der Liste von den bis heute bekannten marinen Isolaten widerspiegelt (Tabelle 1).

3.2 Kultivierungsunabhängiger 16S rRNA-Ansatz

Durch Kultivierung alleine scheint die Frage, welche Spezies im marinen Bakterioplankton vorkommen, nicht beantwortet werden zu können. Der kultivierungsunabhängige rRNA-Ansatz [119, 121], bei dem Bakterien aus der Umwelt gesammelt, deren DNA extrahiert, 16S rDNA mittels spezifischer PCR amplifiziert, kloniert und sequenziert wurden, deckte erstmals die Existenz und Identität von bisher nicht kultivierten Mikroorganismen auf. Seit 1990 wurden aus marinen Umweltproben der küstennahen und offenen See des Atlantiks und Pazifiks zahlreiche 16S rDNA Genbanken erstellt [21, 33, 34, 52, 59, 108, 113, 135, 166, 178]. Sie zeugen von einer großen Diversität des marinen Bakterioplanktons, die das bekannte Spektrum wesentlich erweiterte (Abbildung 5).

Im sogenannten Bakterioplankton wurden nicht nur 16S rRNA Gensequenzen aus der Domäne der Bacteria sondern auch solche der Archaea isoliert [33, 50, 51, 108]. Inwieweit verwandtschaftliche Grade archaeeller Klonsequenzen zu bekannten Archaea Rückschlüsse auf physiologische Eigenschaften zulassen, bleibt bis heute fraglich, da keine kultivierten Vertreter aus dem marinen Pelagial vorliegen.

16S rRNA Gene aus der Domäne Bacteria sind in 16S rDNA Genbanken und einer genomischen Genbank sehr viel stärker repräsentiert [58, 147]. Zirka 80% aller bakteriellen 16S rRNA Gensequenzen fallen in 9 Gruppen (Abbildung 5). Sequenzen des marinen Pikophytoplanktons sind denen von kultivierten *Prochlorococcus* und *Synechococcus* Stämmen (Gattungen der Cyanobakterien) sehr ähnlich [21, 52, 113]. SAR202 repräsentiert eine tief abzweigende Gruppe innerhalb der Grünen-Nicht-Schwefel Bakterien [60]. Die „marine Gruppe A" stellt eine entfernt verwandte Gruppe der Gattungen *Fibrobacter* (Fibrobacteres) und *Chlorobium* (Chlorobia) dar [52, 69]. Innerhalb der δ-Proteobakterien wurde eine unbekannte „marine Gruppe B" entdeckt [50, 178]. Eine bisher nicht kultivierte Gruppe von Actinomyceten wurde von Rappé et al. beschrieben [134]. Innerhalb der α-Proteobakterien wurden drei Gruppen von 16S rRNA Gensequenzen gefunden. Sequenzen der SAR11 Gruppe [43, 59] haben weniger als 82% Ähnlichkeit zu kultivierten Vertretern der gesamten Klasse. SAR116 [113] ist entfernt verwandt (89-92%) mit Vertretern verschiedener Gattungen wie z. B. *Azospirillum*, *Rhodospirillum* und *Magnetospirillum*. Innerhalb der SAR116 Gruppe ist die Sequenzheterogenität derart groß, das sich mehrere Untergruppen bilden. Als einzige seiner Klasse hat SAR83 [113] kultivierte Vertreter, z.B.

Roseobacter spp.. Weniger als 90% Sequenzähnlichkeit zu anderen γ-Proteobakterien haben 16S rDNAs der SAR86 Gruppe [113]. Sie stellen eine eigene phylogenetische Linie ohne kultivierten Vertreter dar.

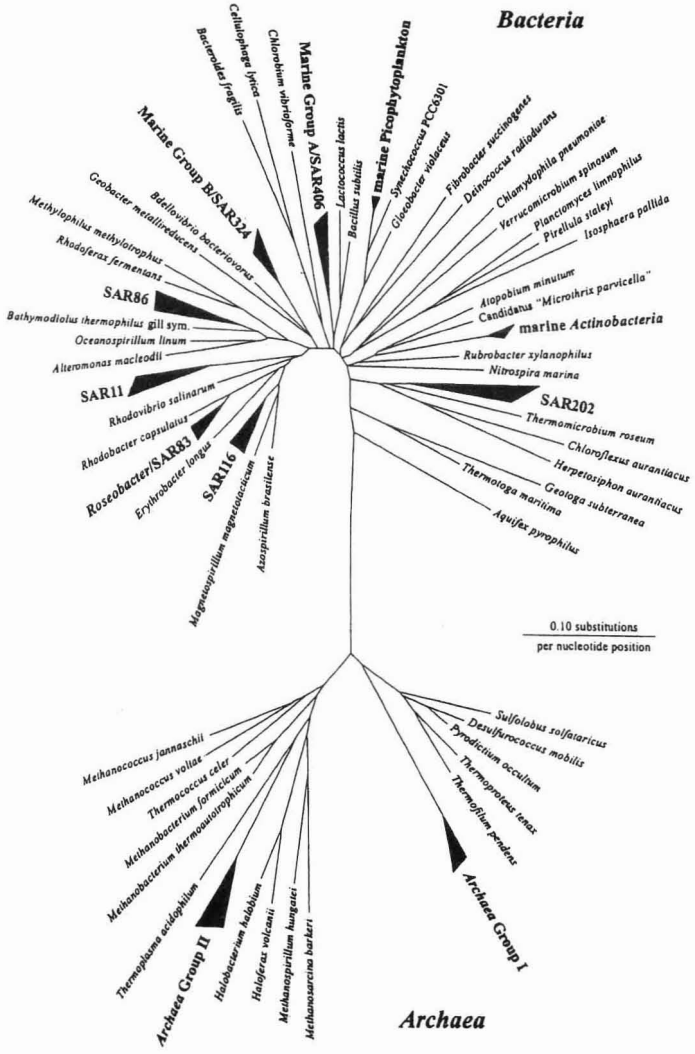


Abbildung 5. Phylogenetischer Stammbaum, der die verbreitesten prokaryontischen 16S rRNA Gensequenz Gruppen des marinen Planktons zeigt [56].

4. Quantifizierung mariner Bakterien

Weder die Häufigkeit von Isolaten in Stammsammlungen noch von Klonsequenzen in Genbanken sagen etwas über die *in situ* Abundanz der jeweiligen phylogenetischen Gruppe aus. Der nächste Schritt nach der Auflistung der bekannten Prokaryonten ist die Bestimmung der relativen Abundanzen der verschiedenen Taxa.

Die 16S rRNA Sequenzinformation ermöglichte die Entwicklung von Oligonukleotidsonden, die spezifisch mit RNA (eventuell auch DNA) der Zielgruppe hybridisieren [119, 160]. Die Hybridisierung erfolgt entweder am gesamten Nukleinsäuregemisch der Umweltprobe oder innerhalb von Einzelzellen und wird autoradiographisch oder epifluoreszenzmikroskopisch sichtbar gemacht.

Aus ersten quantitativen „slot blot" Hybridisierungen des marinen Picoplanktons ging hervor, daß ca. 65 bis zu 97% der Gesamt-rRNA bakterielle rRNA und bis zu ca. 25% archaeelle rRNA darstellt [37, 59]. Die phylogenetische Gruppe SAR11, bislang unkultivierte Vertreter der α-Proteobakterien, formte einen Anteil von bis zu 12% der Gesamt-rRNA [59]. Bekannte in Kultur vorliegende marine Bakterien wie z. B. *Cytophaga* sp., *Sphingomonas* sp. und *Vibrio* sp. wurden mit Anteilen von bis zu 100% an der pelagischen Lebensgemeinschaft quantifiziert. Bei dieser Methode erfolgt die Quantifizierung mittles genomischer Sonden [128] bzw. 16S rDNA Sonden [138], die mit Umwelt-DNA hybridisierten. Der Einzelzellnachweis per Fluoreszenz-*in-situ*-Hybridisierung (FISH) ist aufgrund der schnellen Analyse und der Informationen über Zellgröße, Mikrohabitat etc. attraktiv [6, 36, 62]. Übereinstimmend mit der sensitiven RNA „slot blot" Hybridisierung liegt der so bestimmte Anteil der Eubakterien im marinen Bakterioplankton bei bis zu über 90% [63, 88]. Des weiteren wurden die Abundanzen phylogenetischer Großgruppen bestimmt. Darin zeigte sich, daß die bisher unterrepräsentierte Gruppe der *Cytophaga/Flavobacterium* deutlich häufiger vorkommt als α- und γ-Proteobakterien [63]. Polyribonukleotidsonden mit erhöhter Sensitivität ermöglichen die Anwendung von FISH in oligotrophen Standorten mit geringer bakterieller Aktivität. Mehr als 90% aller DAPI-färbbaren Partikel im offenen Ozean lassen sich damit als Prokaryonten (Bakterien und Archaebakterien) identifizieren [35].

5. Mikrobielle Untersuchungen an planktischen marinen Bakterien

Seitdem Kjelleberg et al. Untersuchungen an hungernden *Vibrio* spp. durchgeführt haben, gehören Vertreter dieser Gattung der γ-Proteobakterien zu den bestuntersuchten marinen Mikroorganismen. Durch Hemmversuche wurden exogene Signalmoleküle für die Induktion der Adaption an den Hunger gefordert [159]. Man beschrieb eine Verkleinerung der Zellgröße in der stationären Phase [78] und Verlust von Speicherstoffen [105] und der Mobilität [163]. Tiefgreifende physiologische Differenzierungen beeinflußten die Lebensfähigkeit, Kultivierung, und Streßresistenz während des Hungerns [92, 117, 118]. Mechanismen der Reaktivierung wurden ebenso untersucht [94, 106]. Hungerversuche wurden auch an anderen marinen Bakterien, wie z.B. *Pseudomonas*, *Salmonella*, *Alteromonas* und *Deleya* Stämmen, durchgeführt [85, 95, 97].

Als Modellmikroorganismus für Oligocarbophilie wurde von Schut et al. [150] ein *Sphingomonas* Stamm (α-Proteobakterien) vorgestellt. Dieser zeigte im Gegensatz zu *Vibrio* sp. keine Miniaturisierung bei Hunger und wies hohe Streßresistenz in eben diesem Zustand auf. Der DNA-Gehalt war niedrig, es lag nur eine Kopie des rRNA Operons vor, der Protein-Gehalt war hoch, und es wurden Mechanismen der zeitgleichen Aufnahme verschiedener Substrate dokumentiert.

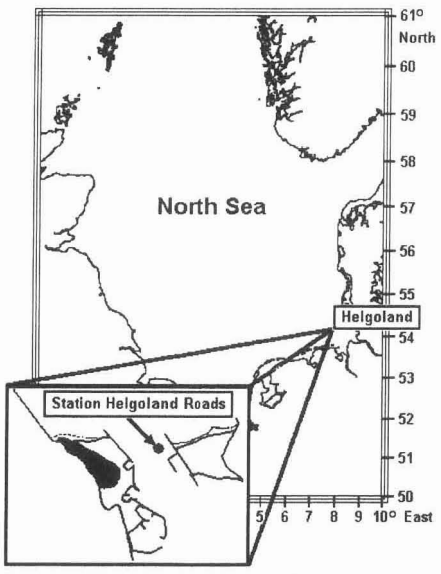
Aus einer Lignin-Anreicherungskultur [68] wurde ein Bakterium des marinen Pelagials gewonnen, das eines der mengenmäßig bedeutensten Pflanzenbestandteile abbaut. Dieser Stamm aus der Gattung *Sagittula* bildete Oberflächenstrukturen aus, die den direkten Kontakt zu Hydrolyseprodukten des Substrates ermöglichten (Verankerungen, Fibrillen) [65]. *Roseobacter* spp., ebenfalls aus der Klasse der α-Proteobakterien stammend, bauen das schwefelhaltige Osmolyt Dimethylsulfoniopropionat (DMSP) der Phytoplankter zu Dimethylsulfid (DMS) ab [66, 98]. Als chemolithoautotrophe Vertreter pelagischer mariner Bakterien wurden Ammonium-oxidierende *Nitrosococcus* spp. identifiziert [5].

6. Zielsetzung der Arbeit

Schwerpunkt dieser Arbeit soll die Kombination von mikrobiologischen Techniken und der kultivierungsunabhängigen 16S rRNA Methode zur Charakterisierung der Gemeinschaft heterotropher Bakterien aus dem marinen Pelagial sein. Damit wird zum einen die Artenvielfalt erfaßt und zum anderen das *in situ* Vorkommen der kultivierten und unkultivierten Mikroorganismen untersucht.

Das Dilemma der „Unkultivierbarkeit" weckt das Interesse, die Lebensstrategie mariner Bakterien zu untersuchen und einen dominanten Vertreter der heterotrophen Lebensgemeinschaft zu kultivieren und zu identifizieren. Die Erkenntnisse von saisonalen Dynamiken wichtiger phylogenetischer Gruppen im Pelagial des Meeres tragen zum besseren Verständnis der Zusammensetzung der heterotrophen Lebensgemeinschaft im Zusammenhang mit anderen biotischen und abiotischen Faktoren bei.

Die Frage des trophischen Status der marinen Bakterien ist nicht eindeutig geklärt. Sollten sich vielleicht ausschließlich copiotrophe Vertreter heterotropher Bakterien [129] in den Kultursammlungen befinden, während obligat oligotrophe fehlen (siehe [151] und Referenzen darin)?



Die Meeresstation Helgoland, liegt in der Deutschen Bucht 60 km vor der Küste (Abbildung 6). Am dortigen Institut mit langer mikrobiologischer Tradition werden routinemäßig seit Jahrzehnten in enger Zusammenarbeit der verschiedenen Abteilungen marinbiologische Daten erfaßt. Die Beprobungsstelle Kabeltonne liegt in mitten der Helgoländer Reede, einer acht Meter tiefe Rinne zwischen der Hauptinsel und der Düne.

Abbildung 6. Lokalisation „Helgoland"

B Ergebnisse und Diskussion

Im folgenden werden die Ergebnisse der einzelnen Publikationen zusammengefaßt und im Zusammenhang diskutiert. Dieser Abschnitt soll allerdings nicht die detaillierten Diskussionen der Einzelpublikationen ersetzen, auf die deshalb hier verwiesen wird. Ausführlicher werden hier Aspekte erläutert, die in den Publikationen nicht oder nur kurz behandelt werden.

1. Diversität und Vorkommen heterotropher Bakterien im marinen Pelagial

Die physiologische Diversität aquatischer Bakterien hinsichtlich ihrer Energie- und Kohlenstoffquellen ist groß. Photoautotrophe Bakterien nutzen Licht und CO_2, chemolithoautotrophe Bakterien verwenden anorganische Verbindungen als Energiequelle und fixieren ebenfalls CO_2. Diesen Primärproduzenten stehen die chemoorganoheterotrophen Bakterien, kurz heterotrophen Bakterien, gegenüber. Sie verwenden organische Verbindungen als Kohlenstoff- und Energiequelle. Die heterotrophen Bakterien sind eine wichtige physiologische Gruppe für den Kohlenstoffkreislauf des marinen Ökosystems. Sie umfassen einen großen Teil der mikrobiellen Lebensgemeinschaft des marinen Pelagials und sind für die biologische Transformation und den Abbau des organischen Materials im Ozean mitverantwortlich.

1.1. Isolierung mariner Bakterien - Stammsammlungen

Die Nährstoffansprüche der meisten heterotrophen marinen Bakterien sind unbekannt. Trotzdem wurden für die Kultivierung heterotropher Bakterien Medien mit komplexen Mischungen von organischem Substrat entwickelt (Referenzen dazu in [151]). Auch solche Medien, die nach Kuznetsov et al. [96] als oligotroph gelten, beeinhalten aufgrund des verwendeten Agars eine vielfach höhere Kohlenstoffkonzentration als sie im natürlichen Seewasser vorkommt. Solche Medien werden bis heute verwendet [17, 20, 66, 67, 99, 128, 133, 166], obwohl die Anzahl koloniebildender Einheiten auf den nährstoffreichen Medien

um Größenordnungen kleiner ist als die Gesamtzellzahl der Ursprungspopulation [82]. Die Verwendung eines künstlichen Seewassermediums [149] in Kombination mit einer verbesserten Kultivierungstechnik von Button et al. [23] und zusätzlicher Variationen der Kultivierungsbedingungen sollte in dieser Arbeit (Publikation 1 und 3) eine größtmögliche kultivierbare Diversität heterotropher Bakterien und die Kultivierung von *in situ* abundanten Vertretern gewährleisten.

Stammsammlung I. Im ersten Teil wurden koloniebildende Bakterien nach morphologischen Kriterien selektiert. Auf diesem Wege wurden insgesamt 145 marine Isolate gewonnen. Davon wurden mit Hilfe von 16S rRNA-gerichteten Oligonukleotidsonden und durch die vergleichende 16S rDNA Sequenzanalyse 122 Isolate den γ-Proteobakterien, elf den α-Proteobakterien, neun Isolate der *Cytophaga/Flavobacterium* Gruppe, zwei Isolate den ε-Proteobakterien und ein Isolat den Actinobakterien zugeordnet.

Mit Hilfe eines genetischen Fingerabdrucks („amplified ribosomal DNA restriction analysis", ARDRA) ließ sich aus der Anzahl der Bandenmuster und Anzahl der Isolate pro Muster in einer „Rarefaction Analyse" abschätzen, inwieweit alle mit den verwendeten Methoden isolierbaren Bakterien erfaßt worden waren. Es zeigte sich, daß hinsichtlich der γ-Proteobakterien vermutlich bereits der überwiegende Teil der mit dieser Strategie kultivierbaren Bakterien isoliert worden war (Abbildung 7). Die „Rarefaction Analyse" sagt voraus, daß mindestens 20 weitere γ-Proteobakterien isoliert werden müßten, um ein neues γ-Proteobakterium mit neuem ARDRA-Muster zu erhalten.

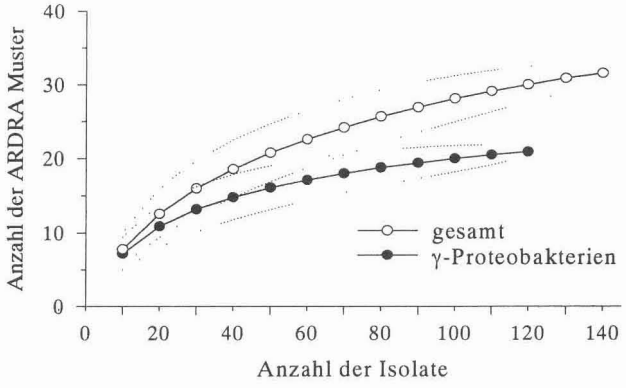


Abbildung 7. Rarefaction Analyse der Isolate aus Stammsammlung I.

Bei den γ-Proteobakterien handelt es sich um Vertreter der Gattungen *Pseudoalteromonas*, *Vibrio*, *Alteromonas*, *Oceanospirillum*, *Halomonas*, *Marinobacter*, *Photobacterium* und *Shewanella*. Die Stämme, die sich der Familie Enterobacteriaceae zugeordnen ließen, wurden als NOR1 (NOR=Nordsee) Gruppe bezeichnet. Stämme, die entfernt der Gattung *Pseudoalteromonas* angegliedert werden konnten, wurden unter der Bezeichnung NOR2 zusammengefaßt (Abbildung 8). Drei Isolate der γ-Proteobakterien

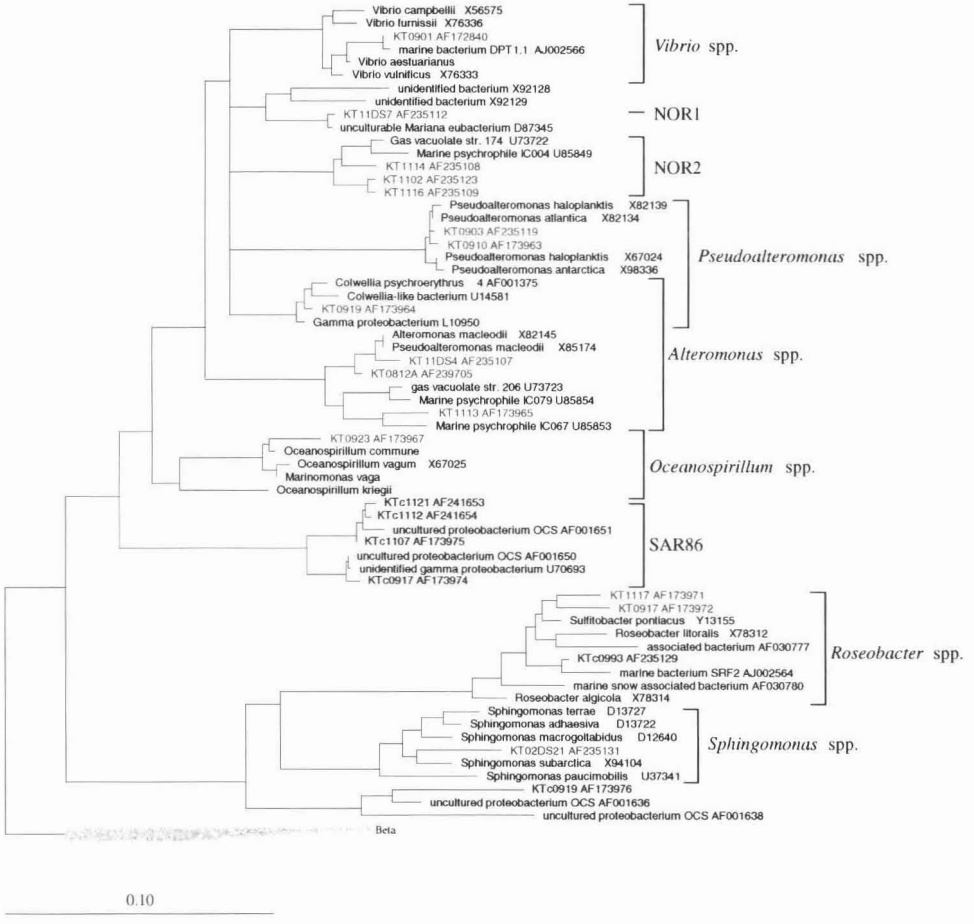


Abbildung 8. 16S rDNA phylogenetischer Stammbaum, der für das marine Pelagial spezifische Gruppen innerhalb der α- und γ-Proteobakterien zeigt. Rot gedruck sind Sequenzen, die von kultivierten Vertretern stammen, blau sind die Sequenzen der Genbank. Der Balken gibt 10% geschätzte Sequenzunterschiede an.

konnten nicht einer bekannten phylogenetischen Linie (Gattung/Familie) zugeordnet werden. Zwei der Isolate wurden mit 16S rDNA Sequenzen von bislang unkultivierten Mikroorganismen und des kultivierten obligat oligocarbophilen Stamms POO-15 [89] unter der Bezeichnung NOR3 als eine phylogenetische Gruppe zusammengefaßt. Ein Isolat aus dieser Arbeit repräsentiert den einzigen Vertreter einer neuen phylogenetischen Linie, die als NOR4 bezeichnet wurde. Interessanterweise hat dieses Isolat aktuell (September 2000) mit 90 bis 92% die höchste 16S rDNA Sequenzähnlichkeit zu Bakterien, die in den oxidativen Teil des Schwefelkreislaufs involviert sind [156], und weist möglicherweise vergleichbare Fähigkeiten auf.

Die im Rahmen dieser Arbeit isolierten α-Proteobakterien sind mit *Roseobacter* spp. und *Sphingomonas* spp. verwandt. (Abbildung 8), Diese Gattungen werden im allgemeinen als marine Bakterien betrachtet [67, 149]. Vertreter der Gattung *Roseobacter* wurden mit dem Abbau schwefelhaltiger Verbindungen, die von marinen phototrophen Organismen produziert werden, in Zusammenhang gebracht [66, 68, 98, 144], während ein *Sphingomonas* Stamm als typisches marines oligocarbophiles Ultramikrobakterium beschrieben wurde [148, 150].

Vertreter der *Cytophaga/Flavobacterium* Gruppe sind sowohl im marinen Benthos [100] als auch im marinen Pelagial [63] als abundant nachgewiesen worden. *Flexibacter* spp. und *Cytophaga* spp., wie sie auch in dieser Arbeit isoliert wurden, wurden bereits mehrfach aus dem marinen Pelagial gewonnen [16, 166]. Bei Vertretern der Gattungen *Flexibacter* und *Cytophaga* handelt es sich um aerobe, mikroaerophile oder fakultativ anaerobe, chemoorganotrophe Bakterien, die in der Lage sind, auch Makromoleküle wie Proteine, Chitin, Pektin, Agar und Stärke abzubauen [29, 139]. Deshalb wurde bei einigen Stämmen dieser Stammsammlung eine Zersetzung des Agars beobachtet.

Bei den im Rahmen dieser Arbeit isolierten ε-Proteobakterien handelte es sich um *Arcobacter* spp.. Vertreter dieser Gattungen wurden zuerst in Kliniken isoliert und als potentiell pathogen beschrieben [104]. *Arcobacter* spp. wurden bisher aus marinen Sedimenten isoliert [168, 169], *in situ* detektiert [100], und häufig als 16S rDNA Klonsequenz gefunden [170]. Vertreter der Actinobakterien hingegen, die als sogenannte Bodenbakterien bekannt sind, konnten in dieser Arbeit aus der Nordsee und von weiteren Standorten des marinen Pelagials isoliert werden [20, 166]. Sie gelten gemeinhin als aerob, physiologisch flexibel und fähig, komplexe organische Substrate abzubauen [83]. Dennoch repräsentieren

die kultivierten Stämme nicht dieselben marinen Actinobakterien wie sie aus 16S rDNA Genbanken erhalten wurden [134].

In dieser Arbeit wurden die Wachstumsbedingungen, wie z.B. Temperatur (4-20°C), *p*H-Wert (*p*H 5,3-8,3), Salinität (25-35‰) und die Verfügbarkeit von Vitaminen und Spurenelementen variiert. Die Ergebnisse der Kultivierung mariner Bakterien veränderten sich nicht signifikant. Möglicherweise ist die Indifferenz hinsichtlich dieser Bedingungen die Ursache für die häufige Isolierung von Bakterien, insbesondere von γ-Proteobakterien [68, 128, 166]. Nur *Roseobacter* spp. der α-Proteobakterien wurden in dieser Arbeit ausschließlich bei *p*H 8,3 und einer Salzkonzentration von 35‰ isoliert. Da *Roseobacter* spp. und Verwandte nicht aus dem Bothnischen Meerbusen der Ostsee [123], wohl aber aus ebenfalls küstennahem Seewasser des Pazifik [166] und Atlantik [67] isoliert wurden, bekräftigt dies die Annahme von Gonzales et al., es handele sich um eine rein marine Gruppe [67].

Stammsammlung II. Für den Aufbau einer zweiten Kultursammlung wurden die Kultivierungsverfahren grundlegend geändert (Publikation 3). Zum einen wurde vermutet, daß langsam wachsende Bakterien durch schnell wachsende koloniebildende Bakterien [172] überwachsen werden könnten. Deshalb wurden, anders als im ersten Teil, die Kolonien nicht nach morphologischen Kriterien selektiert, sondern alle im Binokular (4,5-fache Vergrößerung) sichtbaren Kolonien überimpft. Hierfür wurde eine Art Minispaten entwickelt, mit dem die gesamte Kolonie und das umliegende Agarmedium ausgestochen wurden. Zum anderen wurde in dieser Arbeit, analog dem substratinduzierten Tod [162] von kohlenstofflimitierten Bakterien [91], vermutet, daß phosphat- [22, 28] und/oder stickstofflimitierte Bakterien [174] einer nährstoffinduzierten Wachstumshemmung unterliegen könnten. Deshalb wurden die Konzentrationen von Phosphat und Stickstoff (Ammonium) im künstlichen Seewasser auf das Niveau der natürlichen Phosphat- und Stickstoffkonzentrationen in der Nordsee verringert [64, 71].

Die Modifikation des Kultivierungsmediums und der Überimpfungstechnik hatte eine erhebliche Erhöhung der Kolonieanzahl zur Folge. Während auf Agarplatten mit hohen Phosphat- und Stickstoffkonzentrationen nach zwölf Tagen eine Sättigung bei 20 koloniebildenden Einheiten erfolgte, bildeten sich bei Verwendung des modifizierten Mediums 81 Kolonien. Im Laufe weiterer 24 Tage wurden zusätzliche 46 Kolonien sichtbar, was insgesamt eine Aubeute von 127 koloniebildenden Einheiten ermöglichte. Diese deutliche

Verbesserung der Ausbeute könnte auf die Verringerungen der Phosphat- und Stickstoffkonzentrationen zurückgeführt werden, denn das Angebot von organischen Substraten und deren Konzentrationen von insgesamt 5,7 mg Kohlenstoff pro Liter war in beiden Ansätzen identisch. Die Veränderung der Überimpfungstechnik von Kolonien ermöglichte auch die Ernte von langsam wachsenden Bakterien, denn in Kontrollen waren Agarplatten innerhalb kurzer Zeit (i.d.R. 7-10 Tage) von sich schnell vergrößernden Kolonien überwachsen.

Die taxonomische Zugehörigkeit der Isolate änderte sich signifikant mit der Zeit (Abbildung 9). Schnell wachsende Bakterien konnten den γ-Proteobakterien zugeordnet werden. γ-Proteobakterien stellen nach zwei Tagen einen Anteil von 41% (14 Isolate) der bis dahin gesammelten Kolonien. Des weiteren wurden 21% CF, 9% α-Proteobakterien und 29% andere Stämme isoliert. Eine stichprobenartige Feinanalyse der Stämme zeigte, daß es sich um Vertreter der Gattungen *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Colwellia* und *Photobacterium*, *Polaribacter*, *Cytophaga*, *Flavobacterium*, *Roseobacter* und *Microbacterium* handelte. Kolonien von *Halomonas* spp., *Marinomonas* spp., *Agrobacterium* spp., *Erythromicrobium* spp. und *Flexibacter* spp. entwickelten sich nach sieben Tagen. Nach zwölf Tagen wurden Kolonien von *Shewanella* spp., *Sulfitobacter* spp., *Paracoccus* spp. und des Stamms KT71 sichtbar. Nach 36 Tagen entwickelten sich keine zusätzlichen Kolonien mehr. In der Stammsammlung lagen nun 42% α-Proteobakterien, 29% γ-Proteobakterien, 12% CF und 26% andere Stämme vor.

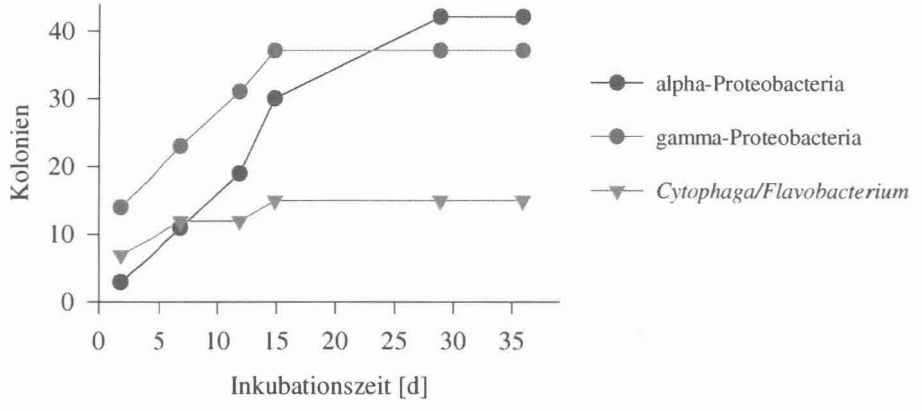


Abbildung 9. Phylogenetische Zuordnung der Isolate durch Fluoreszenz-*in-situ*-Hybridisierung.

Der in dieser Arbeit isolierte Stamm KT71 ist ein γ-Proteobakterium, das keiner bekannten Gattung näher zugeordnet werden kann. Er weist 16S rDNA Ähnlichkeiten von bis zu 94% zu 16S rDNA Klonsequenzen aus Genbanken aus dem Atlantik [52, 135] und aus der Nordsee (Publikation 1) auf. Diese phylogenetische Gruppe wurde im folgenden als NOR5 bezeichnet. Erste Wachstumsversuche zeigten, daß der Stamm KT71 der NOR5 Gruppe ohne bzw. mit 5,7 mg gebundenem Kohlenstoff pro Liter wuchs, jedoch nicht in Medien mit Zusatz von Pepton und Hefeextrakt wie z.B. im klassischen marinen Medium ZoBell 2216.

1.2. 16S rDNA Genbank

Zur Beschreibung der Diversität des marinen Bakterioplanktons wurde neben der Stammsammlung eine 16S rDNA Genbank aus dem Pelagial der Nordsee erstellt (Publikation 1).

In der vorliegenden Genbank wurden 54 zufällig ausgewählte Klone analysiert. Etwa 80% der Klonsequenzen (44 Klone) waren mit solchen der SAR86 Gruppe verwandt (Abbildung 8). Klonsequenzen der SAR86 Gruppe wurden im Laufe der letzten Jahrzehnte auch aus Atlantik-, Pazifik- und Mittelmeerproben enthalten [1, 147, 166]. Obwohl es bis heute keine Isolate gibt, gilt die Gruppe trotzdem als eine der am weitesten verbreiteten Gruppen des marinen Bakterioplanktons [58]. Die vergleichende Sequenzanalyse der in dieser Arbeit erhaltenen 16S rDNA Sequenzen der SAR86 Gruppe bestätigen zwei Untergruppen. Es gibt keine nahe Verwandtschaft zu kultivierten Vertretern der γ-Proteobakterien (16S rDNA-Ähnlichkeit <90%), so daß es unmöglich ist, eine Prognose zur Physiologie dieser Gruppe abzugeben. Gleichweit von der SAR86 Gruppe finden sich z.B. bakterielle Endosymbionten, schwefeloxidierende chemolithotrophe Bakterien und photosynthetische Bakterien [58]. In kultivierungsunabhängigen Untersuchungen der SAR86 Gruppe mit Hilfe der Umweltgenomik („Environmental Genomics") wurde ein bacteriorhodopsinähnliches Protein gefunden [14]. Aber Rückschlüsse auf eine lichtgetriebene Protonentranslokation wie bei Halobakterien sind in diesem Stadium der Untersuchungen sicherlich verfrüht. „Environmental Genomics" hat sich in den U.S.A. als Begriff für die Klonierung und vergleichende Sequenzierung von großen Fragmenten (>50 kbp) von direkt aus der Umwelt gewonnener DNA etabliert [141].

Außer Vertretern der ubiquitären SAR86 Gruppe wurde in der Genbank eine Klonsequenz der NOR5 Gruppe zugeordnet. Auch ihre weltweite Verbreitung zeichnet sich durch die 16S rDNA Klonsequenzen in Genbanken aus Atlantik-, Pazifik- und Mittelmeerproben ab [4, 52, 135]. Wieder machen 16S rDNA Sequenzähnlichkeiten von 93 bis 95% z.B. zu Bakterien aus marinen Schwämmen [4], chemoautotrophen symbiontischen Bakterien aus Muscheln [40], einem psychrophilen Bakterium des antarktischen Eises [70], einem anoxygen phototrophen Eisenoxidierer [39] und einem cycloalkan- (terpen-) abbauenden [175] bzw. sekundärmetabolitproduzierenden *Pseudomonas* sp. [122] eine Prognose über die physiologische Rolle der NOR5 Gruppe schwierig. In diesem Zusammenhang wäre eine detaillierte Untersuchung des Stamms KT71 der NOR5 Gruppe sehr aufschlußreich.

Neben der Dominanz von γ-proteobakteriellen Klonsequenzen in der 16S rDNA Genbank dieser Arbeit fanden sich auch einige, die mit α-Proteobakterien verwandt sind. Zwei der drei weltweit häufigsten Klonsequenz-Gruppen von α-Proteobakterien aus marinem Bakterioplankton, SAR116 und *Roseobacter* [58], wurden ebenfalls in der Nordsee detektiert. Da kein Vertreter der umfangreichen phylogenetischen Gruppe von SAR116 isoliert wurde, gibt es bis heute zur Ökophysiologie dieser Bakterien keine Untersuchungen. Im Gegensatz dazu wurden Vertreter der phylogenetisch kohärenten *Roseobacter* Gruppe von verschiedensten Standorten kultivert (siehe [58] und Referenzen darin). Marine *Roseobacter* Isolate sind physiologisch vielfältig, konnten z.B. mit schwefelhaltigen Verbindungen und mit Lignin angereichert werden [66, 68].

Klonsequenzen aus der *Cytophaga/Flavobacterium/Bacteroides* Gruppe waren in dieser Genbank abwesend. Offenbar diskriminiert der Primersatz *8f*:1542*r* bei der PCR-Amplifikation des 16S rRNA Gens von Umwelt-DNA die Gruppe CFB (siehe [63] und Referenzen darin).

2. *In situ* Abundanz und saisonale Populationsdynamik kultivierter und bisher nicht kultivierter Bakterien des Pelagials der Nordsee

Da es Ziel dieser Arbeit war, Vertreter von Gruppen mit hoher *in situ* Abundanz zu kultivieren, wurde das Vorkommen und die Häufigkeit kultivierter und bisher nicht

kultivierter mariner Bakterien im Pelagial der Nordsee bei Helgoland untersucht (Publikation 1 und 3). Dabei gewährten die jahreszeitlichen Veränderungen der mikrobiellen Gesamtzellzahlen und der Abundanzen phylogenetischer Gruppen im Jahresgang von 1998 einen Einblick in die Populationsdynamik der mikrobiellen Lebensgemeinschaft des Bakterioplanktons (Publikation 3).

Pinhassi et al. [126, 128] und Rehnstam et al. [138] finden, daß nur wenige Species saisonal die Bakterioplanktongemeinschaft dominieren. Im Gegensatz dazu zeigte dieser Jahresgang, daß die mikrobielle Diversität der Nordsee hoch ist. Es gibt sehr viele phylogenetische Gruppen, die *in situ* selten sind. Hiervon befinden sich viele in Kultur. Phylogenetische Gruppen, die numerisch eine Rolle spielen, konnten nur mit verbesserten Kultivierungsmethoden isoliert werden. Inwieweit von vorläufigen mikrobiologishen Charakterisierungen, Aussagen zur Ökophysiologie gemacht werden können, bleibt fraglich; Gruppen wie NOR5 und *Cytophaga/Flavobacterium* weisen eine hohe phylogenetische Tiefe auf, die Raum für verschiedene Anpassungen läßt.

Gesamtzellzahlen und FISH-Detektion. In der Nordsee erfolgte bei ansteigender Wassertemperatur und einsetzenden Phytoplanktonblüten [71] im Frühling (April bis Ende Juni) und im Sommer (Ende Juni bis Anfang September) eine Erhöhung der mikrobiellen Gesamtzellzahl von $0{,}7\times10^6$ auf $1{,}3\times10^6$ bzw. $2{,}1\times10^6$ Zellen pro ml (Abbildung 10). Im Frühling und Sommer wurden 43 bis 91% der Zellen mit Hilfe der Sonde EUB338 der Domäne Bacteria zugeordnet, was frühere Quantifizierungen des bakteriellen rRNA-Anteils an der Gesamt-rRNA bestätigt [59]. Außer im Juni, ließen sich in der Zeit vom April bis Anfang September, die Vertreter der EUB338-detektierten Zellen vorwiegend den phylogenetischen Gruppen von *Cytophaga/Flavobacterium*, α- und γ-Proteobakterien zuordnen. Dabei ist zu beachten, daß α- und γ-Proteobakterien zusammen einen fast gleich großen Anteil am Bakterioplankton darstellen wie Vertreter der *Cytophaga/Flavobacterium* Gruppe.

Gleichzeitig mit dem Zusammenbruch der Frühjahrs-Phytoplanktonblüte erfolgte im Juni eine Verringerung der bakteriellen Gesamtzellzahl. In dieser Zeit konnten im Durchschnitt nur 51% aller Bakterien mit der Sonde EUB338 als Bacteria identifiziert werden. Vertreter der Verrucomicrobia und Planctomycetales haben in dieser Zeit keine Rolle gespielt (Sonde EUB338-II und EUB338-III, [31]). Es könnten sich solche Bakterien entwickelt haben, die nicht für FISH zugänglich sind (Actinobakterien). Neben den Bacteria

könnten außerdem Archaea im Nordseeplankton eine Rolle spielen [74]. In früheren Untersuchungen stellte der archaeelle rRNA-Gehalt bis zu 10% an der Gesamt-rRNA des Epipelagials dar [33, 49, 108, 114].

Nachdem das jährliche Maximum der Gesamtzellzahl im Juli erreicht war, nahm die Zellzahl im August drastisch von 2,1 auf $0{,}7\times10^6$ Zellen pro ml ab (Abbildung 10). Eine erhöhte Zellysefrequenz durch Viren und erhöhter Fraßdruck durch Protisten könnten für den drastischen Zellverlust im August die Ursache sein [53, 131, 158]. Die Zellzahl nahm im Herbst langsam aber kontinuierlich ab und erreichte schließlich den niedrigsten Wert von $1{,}2\times10^5$ Zellen pro ml, was durch die abklingenden Phytoplanktonblüten und fallenden Wassertemperatur begründet sein könnte. In dieser Zeit verringerte sich außerdem die EUB338-Detektionsrate von 75% im Sommer auf 30 bis 50% der Zellen im Winter. Außer EUB338-detektierbarer Bacteria entwickelten sich im Herbst kleine Populationen von Verrucomicrobia und Planctomycetales (ca. 5% der Zellen). Allerdings könnten die niedrigen FISH-Detektionsraten in den Phasen ohne Wachstum (Herbst/Winter) auch auf verringerte RNA-Gehalte der Zellen zurückzuführen sein [90].

Solange die Wassertemperatur im Winter (Januar-März) 4 bis 6°C betrug, blieben die Gesamtzellzahlen auf konstant niedrigem Niveau mit $1{,}2\times10^5$ Zellen pro ml (Abbildung 10). Mehr als zwei Drittel aller EUB338-detektierbaren Zellen (85%) ließen sich in dieser Zeit den

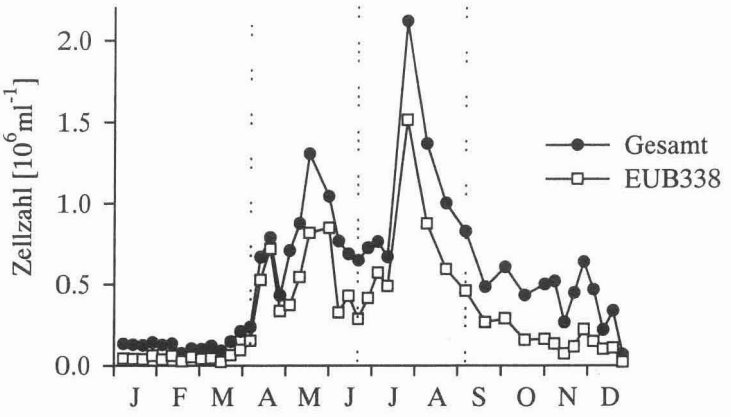


Abbildung 10. Entwicklung der Gesamtzellzahlen und EUB338-Detektionsraten im Jahresverlauf 1998.

Vertretern der *Cytophaga/Flavobacterium* Gruppe (ca. 13%), α-Proteobakterien (ca. 12%) und γ-Proteobakterien (ca. 7%) zuordnen.

In dieser Arbeit sind Vertreter aus jeder dieser drei Gruppen in der Kultursammlung und, mit Ausnahme der *Cytophaga/Flavobacterium* Gruppe, auch in der 16S rDNA Genbank zu finden. Deshalb wurden 16S rRNA-gerichtete Oligonukleotidsonden für häufig repräsentierte phylogenetische Gruppen entwickelt und *in situ* angewendet (Publikation 1 und 3).

Das sukzessive Aufkommen verschiedener Phytoplankter, hauptsächlich von verschiedenen Diatomeen [71], und deren Kohlenstoffinput [15], in Kombination mit der Verringerung von verfügbaren anorganischen Nährstoffen und einem Anstieg der Wassertemperatur, hatte einen Einfluß auf die Zusammensetzung der bakteriellen Lebensgemeinschaft. Ähnliche Zusammenhänge wurden bereits von Garrison et al. bei Untersuchungen des Arabischen Meeres vermutet [56].

Mit der frühen Diatomeenblüte von *Coscinodiscus* spp. bei niedrigen Wassertemperaturen von 7-9°C im April ging eine Zunahme des Anteils der *Cytophaga/Flavobacterium* Gruppe von 10 auf 55% aller Zellen ($4{,}4\times10^5$ Zellen pro ml) einher (Abbildung 11). Im Brackwasser der Ostsee [126] dominiert diese Gruppe ebenfalls im

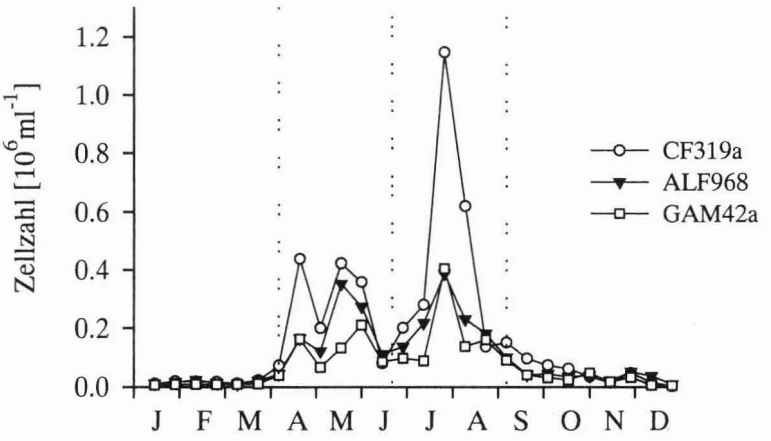


Abbildung 11. Populationsdynamik der *Cytophaga/Flavobacterium* Gruppe, α-Proteobakterien und γ-Proteobakterien im Jahresverlauf 1998.

Frühling das Bakterioplankton. Nachfolgende Blüten von *Rhizosolenia* spp. und *Lauderia* spp. im Mai erfolgten gleichzeitig mit Zunahme der Zelldichten von α- ($3{,}5\times10^5$ Zellen pro ml) und γ-Proteobakterien ($2{,}1\times10^5$ Zellen pro ml) in der Nordsee. Für *Cytophagales*-Verwandte und α-Proteobakterien wurden hohe Wachstumsraten und Enzymaktivitäten bei Diatomeenbüten festgestellt [140]. Bei den jahreshöchsten Wassertemperaturen von 17°C im Sommer (Juli/August) erfolgte 1998 ein weiteres massenhaftes *Lauderia* spp. Aufkommen. Dabei erreichten neben Vertretern der *Cytophaga/Flavobacterium* Gruppe (54% aller DAPI-färbbaren Zellen, $1{,}2\times10^6$ Zellen pro ml) diesmal auch gleichzeitig α- und γ-Proteobakterien (je ca. 19% aller DAPI-färbbaren Zellen; $0{,}4\times10^6$ Zellen pro ml) ihr jährliches Maximum (Abbildung 11).

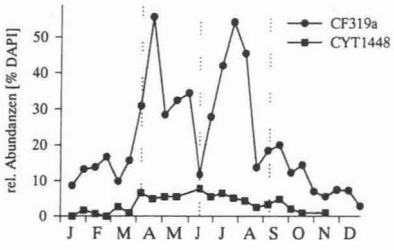


Abbildung 12. Relative Abundanzen von Vertretern der *Cytophaga/Flavobacterium* Gruppe und kultivierten Vertretern der *Cytophaga* spp.

Cytophaga/Flavobacterium. *Cytophaga/Flavobacterium* ist eine wichtige Gruppe der bakteriellen Lebensgemeinschaft im marinen Pelagial [63]. In dieser Arbeit konnte gezeigt werden, daß ihr relativer Anteil an der Gesamtzellzahl im Vergleich zu den Proteobakterien stärkeren saisonalen Schwankungen mit deutlicher Frühlings- und Sommerdominanz während der Phytoplanktonblüten aufweist (Abbildung 12). Mit Hilfe der gattungsspezifischen Sonde CYT1448 wurden kultivierte Vertreter der *Cytophaga* spp. jahreszeitlich quantifiziert. Sie repräsentierten während des Frühlings und Sommers 6 ± 2% aller DAPI-färbbaren Partikel und erreichen ihr saisonales Maximum im Juli/August ($1{,}1\times10^5$ Zellen pro ml) (Abbildung 12). Trotzdem verbleibt ein großer Teil dieser physiologisch vielfältigen Gruppe [139] unbekannt. Diese Gruppe sollte daher in Zukunft intensiver untersucht werden.

α-Proteobakterien. Tendenziell war der Anteil von α- Proteobakterien im Frühling und Sommer größer als im Herbst und Winter (Abbildung 11), doch eine signifikante saisonale Dominanz wurde nicht beobachtet. Die „marine alpha" Gruppe [67] stellt einen großen Anteil der α-Proteobakterien dar (44-100%). Auch diese Gruppe tritt sukzessive im

Frühling (Ende Mai) und Sommer (Juli/August) mit jeweils ca. $2{,}5\times10^5$ Zellen pro ml auf. Mit Hilfe einer spezifischen Sonde (ROS537), die in dieser Arbeit entwickelt wurde, konnten bis zu 22% aller DAPI-färbbaren Zellen (67% aller α-Proteobakterien) der "marinen alpha" Gruppe [67] zugeordnet werden. Die FISH zeigte, daß es sich hierbei um eine morphologisch sehr vielfältige Gruppe handelt, deren Formen von großen Kokken bis zu kleinen Stäbchen reichen. Innerhalb dieser Gruppe repräsentiert kein kultivierter Vertreter einen detektierbaren Anteil am Bakterioplankton. Auch andere α-Proteobakterien, wie *Sphingomonas* spp. wurden nicht mit Hilfe von FISH in der Nordsee gefunden. Im Gegensatz dazu wurde mit Hilfe einer genomischen Sonde ein *Sphingomonas*-Stamm in der Ostsee saisonal mit bis zu 19% Anteilen an der mikrobiellen Gemeinschaft gefunden. Allerdings wird RNA als Zielsequenz für Hybridisierungen der DNA vorgezogen, denn die Wahrscheinlichkeit, daß DNA aufgrund der großen Länge und der Sequenzvielfalt per Zufall mit Sonden hybridisiert, ist groß [54, 59].

γ-Proteobakterien. γ-Proteobakterien wiesen keine saisonale Dominanz auf. Dennoch war der Anteil im Frühling und Sommer größer als im Herbst und Winter. Eine FISH mit spezifischen Sonden für die häufig kultivierten Gattungen *Vibrio* (Sonde GV), *Pseudoalteromonas* und *Alteromonas* (PSA184, ALT1413) und die Gruppen NOR1 (NOR1-56) und NOR2 (NOR2-1453) aus der Klasse der γ-Proteobakterien (Abbildung 8) zeigte, daß die Häufigkeit dieser Gruppen in den meisten untersuchten Proben gering ist. Sie liegt im Bereich des unteren Detektionslimits von ca. 1% aller DAPI-färbbaren Zellen (Publikation 1). Vertreter dieser Gattungen wurden meist in Assoziation mit Kleinstpartikeln (ca. 15-30 μm) gefunden (Abbildung 13). Dies bestätigt Ergebnisse von „marine snow" Analysen [1, 30, 34, 136]. Die so detektierten Bakterien waren größer als 1,2 μm im Durchmesser und wiesen ein intensives Hybridisierungssignal auf. Da im Vergleich zum

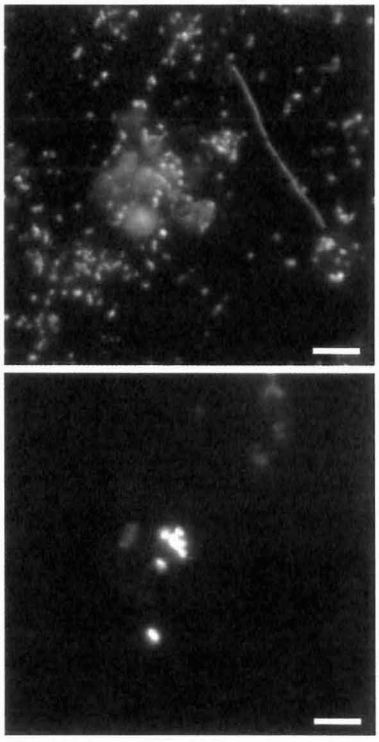


Abbildung 13. Fluoreszenz-*in-situ*-Hybridisierung von Nordsee-Bakterioplankton im September 1998 mit der Sonde G V. Der Balken entsprich 5 μm

umliegenden Freiwasser ein erhöhtes Nährstoffangebot an Partikeln zu erwarten ist [3], könnte angenommen werden, daß partikelassoziierte Bakterien metabolisch aktiver als freilebende Bakterien sind [101]. In traditionellen mikrobiologischen Anwendungen, wie z.B. Komplexmedien in Agar, finden diese aktiven partikelassoziierten Bakterien für sich ideale Bedingungen vor und bilden schnell sichtbare Kolonien.

Neben diesen häufig kultivierten Bakterien wurde erstmalig ein Vertreter der NOR5 Gruppe aus der Klasse der γ-Proteobakterien (Stamm KT71) kultiviert. Die NOR5 Gruppe wurde mittels spezifischer Sonden als dominante phylogenetische Gruppe des Nordsee-pelagials identifiziert. Zellen des Stammes KT71 und weiterer Vertreter der NOR5 Gruppe sind stäbchenförmig (1,6×0,5 μm) und kommen in der Nordsee als freilebende Bakterien vor (Abbildung 14). Diese Gruppe stellte bis zu 60% der γ-Proteobakterien dar. Von Juni bis August repräsentierten sie bis zu 8% (2,2% im Jahresdurchschnitt) aller Zellen des Bakterioplanktons und dominierten somit saisonal die Klasse der γ-Proteobakterien. Gleichzeitig wurden Temperaturen von 12 bis 18°C und die jährlichen Minimalkonzentrationen von anorganischem Phosphor (0,3 bis 0,4 μmol P/l) und Stickstoff (8 μmolN/l) festgestellt [71]. Diese Bedingungen stimmten mit den Kultivierungs-bedingungen überein, die ein Jahr später erfolgreich zur Isolierung des Stammes KT71 aus der NOR5 Gruppe führten.

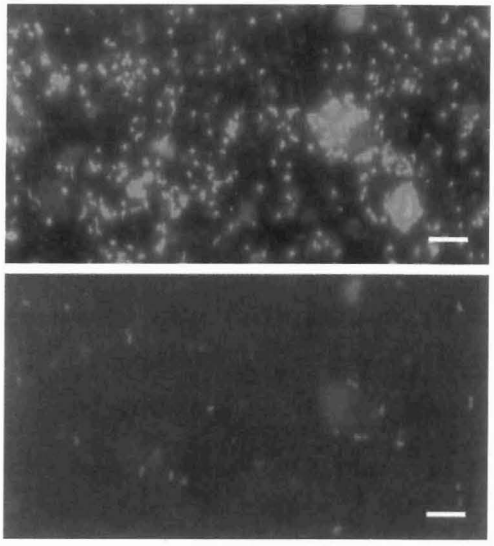


Abbildung 14. Fluoreszenz-*in situ*-Hybidisierung mit der Sonde NOR5-730 (Sommer 1998). Der Balken entspricht 5 μm.

Die Vertreter der ubiquitären SAR86 Gruppe wurden in dieser Arbeit erstmalig mittels spezifischer fluoreszenzmarkierter Sonde *in situ* visualisiert. Es handelt sich um kleine gerade Stäbchen (1×0,5 μm), die freilebend im marinen Pelagial vorkommen, wie es bereits aus dem Fehlen in Genbanken von „marine snow" gefolgert wurde [1]. Die Bakterien der SAR86 Gruppe konnten bis heute nicht kultiviert werden, obwohl sie bis zu 10% aller DAPI-

färbbaren Zellen ausmachen und somit ebenfalls saisonal die γ-Proteobakterien dominieren konnten.

Abschließend bleibt festzustellen, daß aus der Bestimmung der *in situ* Abundanzen sowohl von häufig kultivierten als auch bisher nicht kultivierten phylogenetischen Gruppen hervorgeht, daß bislang nur ein kleiner Teil des Bakterioplanktons identifiziert und phylogenetischen Gruppen zugeordnet werden kann. Die Verwendung moderner Kultivierungstechniken und eine weitere individuelle Verbesserung der Kultivierungsstrategie hat in dieser Arbeit zwar zur Isolierung des dominanten γ-Proteobakteriums geführt, die meisten kultivierten Vertreter sind *in situ* jedoch selten. In stichprobenartigen Überprüfungen konnte gezeigt werden, daß sich Populationszusammensetzungen in der Nordsee bei ähnlichen Phytoplanktonblüten wiederholen können. Dies böte die Möglichkeit, nicht nur gerichtet zu kultivieren, sondern auch eine gezielte Probennahme zu Zeiten hoher Abundanz einer phylogenetischen Zielgruppe durchzuführen. Außerdem könnten dann durch kultiverungsunabhängige Methoden, wie die Erstellung von 16S rDNA und genomischen Genbanken, weitere abundante Gruppen entdeckt werden.

3. Anreicherungskulturen

Ein weiters Ziel dieser Arbeit war es, die Populationsdynamik kultivierter und bisher nicht kultivierter mariner Gruppen in Anreicherungskulturen zu untersuchen. Die Diskrepanz zwischen häufiger Kultivierung von Bakterien und ihrer niedrigen *in situ* Abundanz sollte dabei in Anreicherungen von protistenfreiem Seewasser geklärt werden (Publikation2).
Sobald Seewasser aus dem Meer entnommen und mehrere Stunden oder Tage aufbewahrt wird, unterliegt es Veränderungen. Eine Erhöhung der Gesamtzellzahl und eine Veränderung der phylogenetischen Zusammensetzung der mikrobiellen Lebensgemeinschaft, die insbesondere die γ-Proteobakterien betrafen, bestätigte die Ergebnisse von Fuchs et al. [46]. Im hier durchgeführten Experiment erfolgte im unbehandelten Seewasser innerhalb von 43 Stunden eine Verdoppelung der Zellzahl von 1,6 auf $3x10^6$ Zellen pro ml (Abbildung 15). Der Anteil der γ-Proteobakterien nahm dabei von 13% auf 33% zu. α-Proteobakterien vermehrten sich ausschließlich im substratfreien Seewasser. Doch trotz der Verdoppelung der Zellzahl nahm der relative Anteil von ursprünglich 26% auf 20% ab. Bei Vertretern der

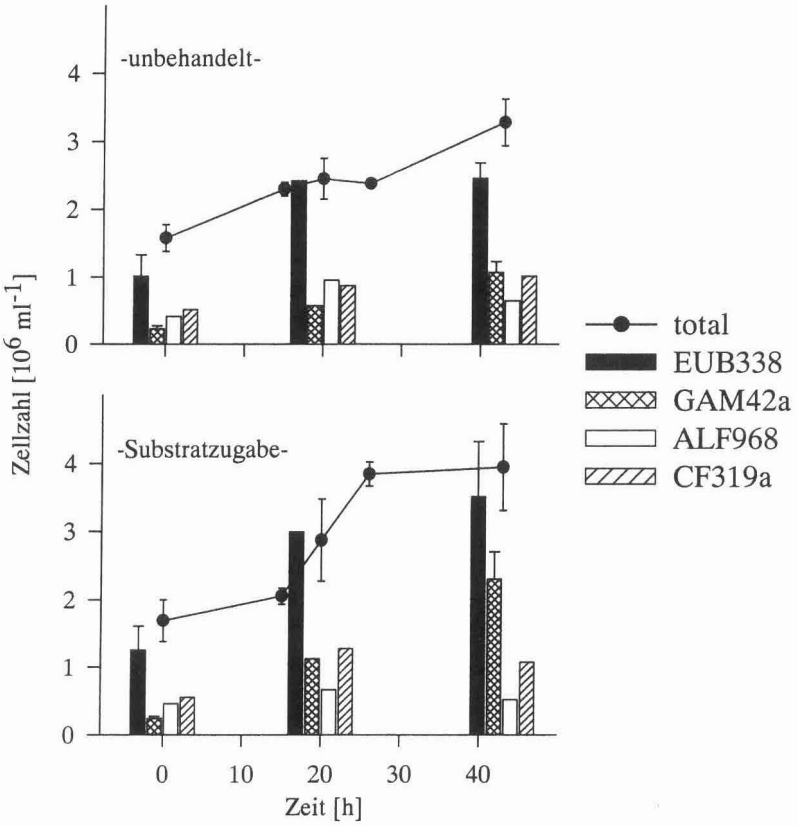


Abbildung 15. Entwicklung der bakteriellen Gesamtzellzahlen (Linien) und der Anteile der mit grupppenspezifischen Sonden hybridisierten Zellen (Balken) während der Inkubation von protistenfreiem Seewasser.

Cytophaga/Flavobacterium Gruppe erfolgte auch eine Verdoppelung der Zellzahl, doch der relative Anteil von 31% blieb in etwa konstant.

Substratzugabe in Form von Monomeren (5,7 mg C/l) verursachte eine noch deutlichere Verschiebung der Zusammensetzung. Die Gesamtzellzahl erhöhte sich nun um den Faktor 2,5 auf 4×10^6 Zellen pro ml. γ-Proteobakterien dominierten mit 58% die mikrobielle Gemeinschaft, während α-Proteobakterien nicht wuchsen und somit nur noch 13% der Bakterien ausmachten. Die Gruppe der *Cytophaga/Flavobacterium* verlor trotz einer Verdoppelung der Zellzahl anteilig 5% (Abbildung 15). Innerhalb der drei phylogenetischen Gruppen, α-Proteobakterien, γ-Proteobakterien und Vertretern der *Cytophaga/Flavobacterium* Gruppe erfolgten Änderungen in der Anzahl und Zusammensetzung der jeweiligen Population in Abhängigkeit von der Substratkonzentration. Da γ-Proteobakterien nicht nur häufig

kultiviert wurden, sondern auch in diesem Experiment deutliche Veränderungen der mikrobiellen Lebensgemeinschaft verursachten, wurden sie näher untersucht.

γ-Proteobakterien. Der Anteil der γ-Proteobakterien nahm im unbehandelten Seewasser von 13% auf 33% zu, bei Substratzugabe sogar auf 58%. Die Verschiebung der phylogenetischen Zusammensetzung erfolgte sowohl in der Gemeinschaft als auch innerhalb der γ-Proteobakterien. Die detailliertere Aufschlüsselung der Zusammensetzung der γ-Proteobakterien ergab, daß direkt nach der Probennahme Vertreter der unkultivierten SAR86 Gruppe (Abbildung 16) und ein erstmals in dieser Arbeit kultivierter Stamm der NOR5 Gruppe (KT71, Daten nicht gezeigt) dominierten. Gleichzeitig befanden sich andere häufig kultivierte Bakterien numerisch am Detektionslimit. Innerhalb von zwei Tagen vergrößerte sich im unbehandelten Seewasser der Anteil der *Alteromonas/Colwellia*-Population von 1,5% auf 20% der Gesamtbakterien (64% der γ-Proteobakterien) und überstieg damit den Anteil der SAR86 und NOR5 Gruppe, die aber in ihren absoluten Zellzahlen von ca. 1,8 bzw. $0{,}6\times10^5$ Zellen pro ml konstant blieben. Ebenso wurden *Oceanospirillum* spp. nicht angereichert, obwohl es in dieser Arbeit auf Agarplatten kultiviert wurde (Publikation 1).

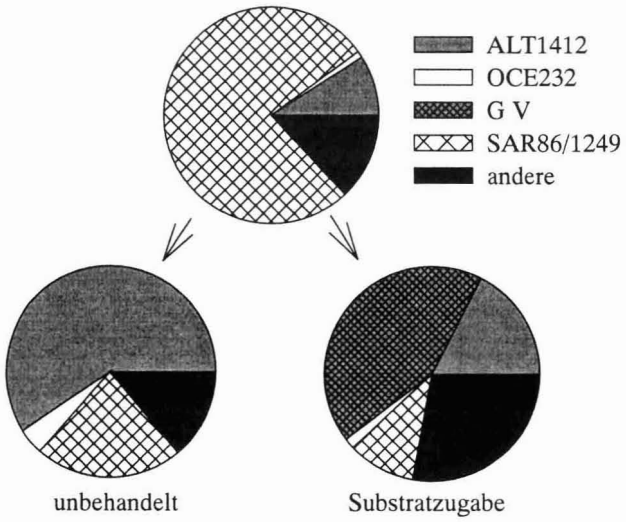


Abbildung 16. Veränderung der taxonomischen Zusammensetzung der γ-Proteobakterien während der Inkubation von unbehandeltem bzw. substratsupplementiertem (5,7 mg C/l) Seewasser.

Die Substratzugabe verursachte eine differenzierte Verschiebung der Zusammensetzung. Die Vergrößerung der Population der γ-Proteobakterien war hauptsächlich auf die Vergrößerung der *Vibrio*-Population (25% der Gesamtbakterien), die ohne Substrat nicht wuchs, und weniger auf die der *Alteromonas/Colwellia*-Population (10% der Gesamtbakterien) zurückzuführen. Wiederum erfolgten keine Veränderungen in der Anzahl der Bakterien der SAR86 und NOR5 Gruppe und auch *Oceanospirillum* spp. wuchsen nicht im substratangereicherten Seewasser. Diese Beobachtung zeigte gleichzeitig, daß weder Bakterien der SAR86, der NOR5 Gruppe und der Gattung *Oceanospirillum* dem sogenannten substratinduzierten Tod [162] erlagen. Möglicherweise wurden diese nicht wachsenden Bakterien durch antibakterielle Faktoren, die von schnell wachsenden Bakterien sezerniert wurden, im Wachstum inhibiert. Dies wurde für *Alteromonas* spp. gezeigt, die diese Verbindungen zur Koordination des Wachstums der gesamten Population und gleichzeitig zur Verteidigung gegen Konkurrenten einsetzen [167].

Aktivierung von Bakterien. Weil die Hybridisierung des fluoreszenzmarkierten Oligonukleotids an die 16S rRNA des Ribosoms erfolgt, kann die Intensität des Signals ein Maß für die Kapazität der Proteinbiosynthese und damit für die metabolische Aktivität sein [6, 90]. Beispielsweise zeigten kultivierte Stämme der Gattungen *Vibrio*, *Alteromonas* etc. und der Gruppe NOR5 ein starkes Signal. Allerdings konnten Fuchs et al. [45] am Modellorganismus *Escherichia coli* zeigen, daß das Zielmolekül der Hybridisierung, die 16S rRNA, aufgrund ihrer Sekundär- und Tertiärstruktur Bindungsstellen unterschiedlicher Zugänglichkeit hat, die die Fluoreszenzintensität beeinflussen. So z. B. haben *Oceanospirillum* spp. schwache FISH-Signale, sind aber möglicherweise aktiv (Publikation 1).

Bakterien der Gattungen *Vibrio* und *Alteromonas/Colwellia* kommen im Vergeich zu ihrer *in situ* Abundanz in den Kultursammlungen dieser Arbeit überdurchschnittlich häufig vor. Da Ouverney et al. die FISH-Detektionsrate durch methodische Verbesserungen erhöhen konnten [120], lag zunächst die Vermutung nahe, daß inaktive, d.h. ruhende ("dormant") oder hungernde ("starving") Zellen in dieser Arbeit durch FISH nicht detektiert wurden. Deshalb wurde das Antibiotikum Nalidixinsäure eingesetzt. Es inhibiert die prokaryontische DNA-Replikation, erlaubt aber das Größenwachstum der Zellen [93]. Damit konnte gezeigt werden, daß keine zusätzlichen *Vibrio* sp., *Alteromonas* sp. und *Colwellia* sp. aus potentiell inaktiven Populationen detektiert wurden. Im Gegenteil, in dieser Arbeit wurde übereinstimmend mit

Flärdh et al. [44] gezeigt, daß Bakterien wie z.B. *Vibrio* sp., *Alteromonas* sp. etc. auch in Hungerphasen „überschüssige" Ribosomen enthalten und somit auch dann detektierbar sind.

Die Verwendung des Antibiotikums hatte allerdings ein Absterben der Bakterien der SAR86 und NOR5 Gruppe zur Folge. Möglicherweise ist Nalidixinsäure für sie toxisch [2]. Hingegen vermehrten sich resistente [24] Vertreter von *Vibrio* und *Alteromonas/Colwellia* nach 20 Stunden und überwuchsen zügig die anderen Bakterien.

Lebensstrategien mariner γ-Proteobakterien. Zum einen gibt es schnell wachsende häufig kultivierte Bakterien wie *Vibrio* spp. bzw. *Alteromonas* spp. und *Colwellia* spp., zum anderen gibt es nicht kultivierte bzw. nur mit großem Aufwand kultivierbare Bakterien, wie Vertreter der SAR86, der NOR5 Gruppe und *Oceanospirillum*. Nach heutigem Kenntnisstand würde man diese Bakterien als *r*- bzw. *K*-Strategen, also als copiotrophe [75] und oligotrophe Bakterien [129], betrachten. Doch die Zuordnung geht über die *r*- und *K*-Selektion der mikrobiellen Ökologie [8] hinaus.

Vibrio bzw. *Alteromonas/Colwellia* als potentielle *r*-Strategen zeichnen sich durch eine hohe Streßresistenz und Toleranz gegenüber inhibierenden Substanzen aus. Flärdh et al. [44] konnten zeigen, daß Ribosomen bei kohlenstofflimiterten, hungenden *Vibrio* sp. im Überschuß vorhanden sind, was sie möglicherweise in die Lage versetzt, schnell auf sich verändernde Bedingungen hinsichtlich des Substratangebots zu reagieren. Innerhalb dieser Gruppe differenzierten sich *Alteromonas* sp. heraus, die schon bei niedrigen Substratkonzentrationen wuchsen. Währenddessen dominierte *Vibrio* sp. umso stärker bei Substratsupplementation. Scheinbar muß eine Schwellenwertkonzentration eines Substrates erreicht sein, um das Wachstum in Gang zu setzen.

Vertreter der SAR86, der NOR5 Gruppe und der Gattung *Oceanospirillum* entsprachen dem Muster eines *K*-Strategen. Möglicherweise erfolgte kein Wachstum im Seewasser, da hier bei der Vorfiltration eine erhöhte Konzentration von Kohlenstoffverbindungen aufgrund der Zerstörung von Algen zu erwarten war [42]. In der Umwelt sind sie möglicherweise an permanent niedrige Nährstoffkonzentrationen angepaßt.

4. Ausblick

Bis heute ist der Stamm KT71 der phylogenetischen Gruppe NOR5 der einzige in Kultur befindliche und *in situ* häufige marine Mikroorganismus des Pelagials der Nordsee. Deshalb wäre es interessant, seine Ökophysiologie zu erforschen. Dafür sollte der Stamm allgemein charakterisiert werden. Außerdem könnten in Experimenten (z. B. Konkurrenz, Hunger) auf molekularer Ebene die Expression funktioneller Gene untersucht werden.

Um weitere *in situ* häufige Bakterien anzureichern, könnten solche Experimente an diese Arbeit anknüpfen, die das Überwachsen der mikrobiellen Gemeinschaft durch schnell wachsende Bakterien auch in Flüssigkulturen verhindern. Dies könnte in Zukunft möglicherweise durch den Einsatz von Protisten bewerkstelligt werden. Protisten können größenselektiv Bakterien aufnehmen [109] aber auch solche Bakterien, die aktiv wachsen, bevorzugen [152]. Bei Plattierungen von Bakterien könnten weitere Optimierungen der Kultivierungsbedingungen zur Isolierung von bisher nicht kultivierten Bakterien führen. So z. B. ist die *Cytophaga/Flavobacterium* Gruppe wenig erforscht. Die mikrobiologischen Isolierungsversuche und Klonsequenzen aus 16S rDNA Genbanken haben nur einen kleinen Teil dieser Gruppe identifiziert. Hinsichtlich ihrer großen Bedeutung im marinen Sediment [100, 137] und, wie diese Arbeit zeigte, ihrem hohen Vorkommen im Pelagial, stellen sie eine interessante Gruppe mit viel Potential für zukünfige Untersuchungen dar.

Diese Arbeit hat gezeigt, wie wichtig das „Monitoring" des marinen Bakterioplanktons ist. Deshalb sollten solche Jahresgänge nicht nur weitergeführt werden, sondern ebenfalls bisher gut untersuchte Systeme wie die Sargasso See (Atlantik) in ähnliche Untersuchungen einbezogen werden. Möglicherweise wäre man später in der Lage, Vorraussagen zur Populationsdynamik zu machen und gezielt Bakterien aus dem Meer für die gerichtete Kultivierung zu sammeln.

Die Kultivierung weiterer bisher unbekannter mariner Bakterien ist die Grundlage für die Erforschung und Nutzbarmachung von bakteriellen Sekundärmetaboliten. So wurden z.B. schon aus den Reihen der γ-proteobakteriellen Gattungen *Pseudoalteromonas*, *Alteromonas*, *Streptomyces* und *Vibrio*, Sekundärmetabolite mit cytotoxischen, antiviralen, antibakteriellen, antitumoralen und algizidalen Wirkungsweisen beschrieben [9, 41, 102, 115, 144, 167](siehe [17] und Referenzen darin).

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Teil II

Publikationen

A Publikationsliste mit Erläuterungen

Diese Dissertation beruht auf drei Publikationen. Die Beiträge der verschiedenen Autoren zu den Publikationen werden im Folgenden erläutert.

1. **Heike Eilers, Jakob Pernthaler, Frank Oliver Glöckner, and Rudolf Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Applied and Environmental Microbiology **66**:3044-3051.

   Entwicklung des Konzepts von H.E., Durchführung der Experimente von H.E., Stammbaum-Berechnung von H.E. mit Unterstützung von FO.G., Erstellen des Manuskripts von H.E. unter redaktioneller Mitarbeit von J.P. und R.A.

2. **Heike Eilers, Jakob Pernthaler, and Rudolf Amann.** 2000. Succession of pelagic marine bacteria during an enrichment: A close look on cultivated-induced shifts. Applied and Environmental Microbiology **66**: 4634-4640.

   Entwicklung des Konzepts von H.E. und J.P., Durchführung der Experimente von H.E. mit Unterstützung von J.P., Erstellen des Manuskripts von H.E. unter redaktioneller Mitarbeit von J.P.

3. **Heike Eilers, Jakob Pernthaler, Jörg Peplies, Frank Oliver Glöckner, Gunnar Gerdts, Christian Schütt, and Rudolf Amann.** Seasonal dynamics of cultured and uncultured pelagic bacteria in the North Sea. Submitted to Applied and Environmental Microbiology.

   Entwicklung des Konzepts von H.E. und J.P., Durchführung der Experimente von H.E., J.P. und J.P. mit Unterstützung von G.G. und C.S., Stammbaum-Berechnung von H.E. und J.P. mit Unterstützung FO.G., Erstellen des Manuskripts von H.E. unter redaktioneller Mitarbeit von J.P.

1.

Culturability and *in situ* abundance of pelagic bacteria from the North Sea

Heike Eilers, Jakob Pernthaler, Frank Oliver Glöckner, and Rudolf Amann

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Culturability and In Situ Abundance of Pelagic Bacteria from the North Sea

HEIKE EILERS, JAKOB PERNTHALER,* FRANK OLIVER GLÖCKNER, AND RUDOLF AMANN
Max-Planck-Institut für marine Mikrobiologie, D-28359 Bremen, Germany

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The culturability of abundant members of the domain *Bacteria* in North Sea bacterioplankton was investigated by a combination of various cultivation strategies and cultivation-independent 16S rRNA-based techniques. We retrieved 16S rRNA gene (rDNA) clones from environmental DNAs and determined the in situ abundance of different groups and genera by fluorescence in situ hybridization (FISH). A culture collection of 145 strains was established by plating on oligotrophic medium. Isolates were screened by FISH, amplified ribosomal DNA restriction analysis (ARDRA), and sequencing of representative 16S rDNAs. The majority of isolates were members of the genera *Pseudoalteromonas*, *Alteromonas*, and *Vibrio*. Despite being readily culturable, they constituted only a minor fraction of the bacterioplankton community. They were not detected in the 16S rDNA library, and FISH indicated rare (<1% of total cell counts) occurrence as large, rRNA-rich, particle-associated bacteria. Conversely, abundant members of the *Cytophaga-Flavobacteria* and gamma proteobacterial SAR86 clusters, identified by FISH as 17 to 30% and up to 10% of total cells in the North Sea bacterioplankton, respectively, were cultured rarely or not at all. Whereas SAR86-affiliated clones dominated the 16S rDNA library (44 of 53 clones), no clone affiliated to the *Cytophaga-Flavobacterum* cluster was retrieved. The only readily culturable abundant group of marine bacteria was related to the genus *Roseobacter*. The group made up 10% of the total cells in the summer, and the corresponding sequences were also present in our clone library. Rarefaction analysis of the ARDRA patterns of all of the isolates suggested that the total culturable diversity by our method was high and still not covered by the numbers of isolated strains but was almost saturated for the gamma proteobacteria. This predicts a limit to the isolation of unculturable marine bacteria, particularly the gamma-proteobacterial SAR86 cluster, as long as no new techniques for isolation are available and thus contrasts with more optimistic accounts of the culturability of marine bacterioplankton.

ZoBell's landmark paper on the taxonomy and abundance of marine bacteria (60) essentially defined this group for a long time. The species then most frequently cultured from marine water samples belonged to the genera *Pseudomonas*, *Vibrio*, *Spirillum*, *Achromobacter*, *Flavobacterium*, and *Bacillus*, and these were assumed to be dominant in marine waters.

The discrepancy between direct microscopic enumeration and plate counts of bacteria was first pointed out by Jannasch and Jones (27). They attributed it to the presence of bacteria in aggregates, to selective effects of the media used, and to the presence of inactive cells. In 1982, Colwell and coworkers developed the viable-but-nonculturable hypothesis (59). Ferguson et al. showed that >99.9% of the natural bacterioplankton community in seawater could not be cultured on Marine Agar 2216 (13). Cultivation failed to solve the discrepancy for a long time. It was left to the cultivation-independent rRNA approach, most notably to 16S rRNA gene (rDNA) clone libraries, to reveal the high additional diversity of marine bacterioplankton communities (7, 11, 15–17, 35, 42). In the Atlantic and Pacific Oceans, most of the sequences clustered within the alpha (e.g., SAR11 and SAR116) and gamma (e.g., SAR92 and SAR86) subclasses of *Proteobacteria* and two novel groups of *Archaea* were found. 16S rDNA sequences of previously isolated marine bacteria (e.g., *Pseudomonas*, *Rhodobacter*, and *Arthrobacter* spp.) were also occasionally retrieved, but their in situ abundances remained unknown (6, 55).

In view of the difficulty of isolating common marine bacteria, dilution culture methods were applied (10). This led to strategies for optimizing viability determinations and eventually to the pure culture of, so far, only one strain of a probably typical marine oligocarbophilic bacterium (48). In contrast, based on DNA-DNA hybridization of the genomic DNAs of isolates obtained with the traditional ZoBell medium against community DNA, it has been suggested that readily culturable bacteria are abundant in the marine water column (21, 22, 40, 44). The aim of this study was to address these discrepancies by evaluating which microorganisms in the North Sea bacterioplankton are readily culturable. For this, we combined cultivation on defined oligotrophic medium with cloning of PCR-amplified environmental 16S rDNAs and fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Sampling and fixation. In September and November 1997 and February and August 1998, surface water samples were collected at a 1-m depth in acid-washed and seawater-prerinsed 50-liter polyethylene containers. The sampling station Helgoland Roads (54°09'N, 7°52'E) is near the island of Helgoland, approximately 50 km offshore in the German Bay of the North Sea. Samples were stored at 4°C and further processed within approximately 5 h.

For DNA extraction, prefiltered picoplankton (cellulose nitrate filter; diameter, 47 mm; pore size, 5 μm; Sartorius AG, Göttingen, Germany) was collected in September 1997 and unfiltered picoplankton was collected in November 1997 by filtration of 1 to 3 liters of water on white polycarbonate filters (diameter, 47 mm; pore size, 0.2 μm; type GTTP2500; Millipore, Eschborn, Germany).

For FISH, 10- to 100-ml samples of unfiltered seawater were fixed with formaldehyde (final concentration, 2% [wt/vol]) for 30 min at room temperature, collected on white polycarbonate filters (diameter, 47 mm; pore size, 0.2 μm; type GTTP2500; Millipore), and rinsed with double-distilled water. Filters were stored at −20°C until further processing.

Enrichment and isolation of marine microorganisms. For cultivation, synthetic seawater was prepared as described by Schut et al. (48). Trace elements and vitamins were added separately. A mixture of monomers (alanine, L-aspartate, DL-leucine, L-glutamate, L-ornithine, and DL-serine [all at 1 μM];

* Corresponding author. Mailing address: Max-Planck-Institut für marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49 421 2028 940. Fax: 49 421 2028 580. E-mail: jperntha@mpi-bremen.de.

TABLE 1. Oligonucleotide probes used for FISH

Probe	Specificity	Probe sequence (5′–3′)	Target site[a] 16S rRNA positions	% FA[b]	Source or reference
ALT1413	*Alteromonas, Colwellia*	TTTGCATCCCACTCCCAT	1413–1430	40	This study
G Rb	*Rhodobacter, Roseobacter*	GTCAGTATCGAGCCAGTGAG	645–626	30	18
G V	*Vibrio*	AGGCCACAACCTCCAAGTAG	841–822	30	18
NOR1-56	NOR1 lineage	TTACCGCTCGGACTTGCA	56–73	20	This study
NOR2-1453	NOR2 cluster group A	GGTCATCGCCATCCCC	1453–1468	30	This study
OCE232	*Oceanospirillum*	AGCTAATCTCACGCAGGC	232–249	40	This study
PSA184	*Pseudoalteromonas, Colwellia*	CCCCTTTGGTCCGTAGAC	184–210	30	This study
SAR86-1249	SAR 86 cluster[c]	GGCTTAGCGTCCGTCTG	1249–1265	50	This study
SPH120	*Sphingomonas*	GGGCAGATTCCCACGCGT	120–137	30	36

[a] *E. coli* numbering (8).
[b] Percent (vol/vol) formamide (FA) in FISH buffer.
[c] Reference 35.

glucose, fructose, galactose, glycolate, succinate, and mannitol [all at 10 μM]; and acetate, lactate, ethanol, and glycerol [all at 15 μM]) was added as a substrate.

The cultivation conditions of this basic approach were modified, e.g., by varying the pH (5.7 and 8.3) or salinity (25 and 35 g of NaCl per liter), by the absence of vitamins and trace elements, and by replacing the monomers with a mixture of polymers (chitin, cellulose, xylan, and pectin [1 g of each per liter] and starch [5 g/liter]).

Aliquots (100 μl) of unfiltered and filtered (cellulose nitrate filter; diameter, 47 mm; pore sizes, 5.0, 1.2, 0.45, and 0.22 μm; Sartorius AG) seawater were either directly spread on plates containing 1% (wt/vol) agar (Difco) or preincubated in a dilution series of the corresponding medium. Colonies were selected randomly from agar plates and subcultured at least three times under the same conditions.

16S rDNA clone library construction. Total nucleic acids were extracted by procedures described by Tsai and Olson (56) from the filters prepared in September and November 1997. Bacterial 16S rRNA primers 8*f* (5′-AGAGTTTG ATCMTGGC-3′) and 1542*r* (5′-AAAGGAGGTGATCCA-3′) were used to amplify almost full-length 16S rDNAs from total community DNA (9) by PCR (46). The amplified rDNA was inserted into the pGEM-T vector (Promega Corp., Madison, Wis.) in accordance with the manufacturer's instructions. Competent *Escherichia coli* JM109 cells (Promega) were transformed and screened for plasmid insertions by following the manufacturer's instructions.

Sequencing and phylogenetic analysis. Plasmid DNAs from selected 16S rDNA clones and amplified 16S rDNAs from isolates were sequenced by *Taq* Cycle Sequencing and universal 16S rRNA-specific primers using an ABI377 (Applied Biosystems, Inc.) sequencer. All sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (32), which compares the phylogenetic affiliations of the 5′ and 3′ ends. Sequence data were analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). A phylogenetic tree was reconstructed using neighbor-joining, maximum-parsimony, and maximum-likelihood analyses. Only sequences at least 90% complete were used for tree construction. Alignment positions at which less than 50% of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA, which cause mistakes in tree topology.

Amplified ribosomal DNA restriction analysis (ARDRA). Purified (QIAquick Purification Kit; Qiagen, Hilden, Germany), amplified 16S rDNAs (approximately 1 μg) from all of the isolates were digested with 7.5 U of the restriction endonuclease *Hae*III (Promega) for 3 h at 37°C. The fragments were analyzed by polyacrylamide gel electrophoresis, and restriction patterns were compared visually. The diversity of the isolates compared to total culturable diversity by our approach was analyzed by rarefaction analysis (51). This was performed for all isolates and for all isolated members of the gamma proteobacteria. Rarefaction curves were produced using the analytical approximation algorithm of Hurlbert (26), and 95% confidence intervals were estimated as described by Heck et al. (24). Calculations were performed using the freeware program "a RarefactWin" (http://www.uga.edu/~strata/Software.html).

Cell counts, FISH, and oligonucleotide probe design. Total bacterioplankton counts were determined by epifluorescence microscopy of acridine orange-stained cells (25). Screening of isolates and determination of North Sea bacterioplankton community structure were performed by FISH. Cells from a single colony of each isolate were transferred to Teflon-coated microscope slides and immobilized by air drying. After dehydration and fixation with 50, 80, and 96% (wt/vol) ethanol, cells on slides and on filter sections were hybridized with oligonucleotide probes EUB338 (3), ALF968 (36), GAM42a (34), and CF319a (33). Counterstaining with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) and mounting for microscopic evaluation were performed as described previously (3, 19).

Oligonucleotide probes ALT1413, PSA184, SAR86-1249, NOR1-56, NOR2-1453, and OCE232 (Table 1) were designed using the PROBE_FUNCTIONS tool of the ARB software package. Their specificity was evaluated with the PROBE_MATCH tool of the ARB package against the rRNA database of the Technical University of Munich (release 12/98). CY3-labeled probes were synthesized by Interactiva (Ulm, Germany). Hybridization conditions for the newly designed probes were optimized by varying the concentration of formamide (37).

Nucleotide sequence accession numbers. The 16S rDNA sequences of the isolates and clones generated in this study were deposited in GenBank under accession numbers AF172840, AF173962 to AF173976, AF235107 to AF235131, AF239705 to AF239707, AF241653, and AF241654.

RESULTS

Diversity of isolated strains. In September and November 1997 and February and August 1998, a total of 145 strains were isolated from North Sea surface water. Initial screening by FISH showed the hybridization of 9 with probe CF319a, 11 with ALF968, 110 with GAM42a, and 15 with none of the group-specific probe but only with EUB338. Sequencing and phylogenetic analysis of the latter 15 strains revealed that 1 was related to gram-positive bacteria with a high G+C DNA content (*Arthrobacter* spp.) and two strains were affiliated with epsilon proteobacteria (*Arcobacter* spp.). The remaining 12 strains were found to be gamma proteobacteria of the genera *Pseudoalteromonas* (10 strains) and *Alteromonas* (2 strains). Representatives from two out of three *Pseudoalteromonas* ARDRA patterns (10 isolates) and from one out of five *Alteromonas* ARDRA patterns (2 isolates) were not detected by probe GAM42a, although they are gamma proteobacteria. Sequencing of the 23S rDNAs of two representatives of each group revealed a single base change from C to T at position 1032 in helix 42 (31) (data not shown), which is the target site of probe GAM42a.

Subsequently, selected clones of each ARDRA pattern were sequenced. Altogether, 95 (35 nearly complete and 60 partial) 16S rRNA sequences of isolated strains were determined, including at least one full and several partial sequences for each ARDRA pattern. Identical *Hae*III ARDRA patterns exhibit highly similar full-length or partial 16S rDNA sequences. This was experimentally verified for frequent isolates, i.e., the NOR2 cluster and the genera *Vibrio* and *Oceanospirillum* (data not shown). Comparative sequence analysis indicated that 142 of 145 strains were closely related to known marine bacteria (16S rRNA similarity, >93%; Table 2). Only three strains grouping within two gamma-proteobacterial clusters (referred to as NOR3 and NOR4 in Table 2) have no known close cultured relative. There was evidence of an effect of filtration and changes in cultivation conditions on the species composition of isolates. Prefiltration of water with a 1.2-μm filter fa-

TABLE 2. Frequency and phylogenetic affiliations of North Sea isolates[a]

Phylogenetic group	No. (%) of isolates	Next relative to isolates[b]	% 16S rRNA similarity
Bacteria	145 (100)		
Cyt/Fla[c]	9 (6.2)		
Flexibacter	2	*Cytophaga marinoflava* M58770	98.0
	2	Marine bacterium strain E110 AF052742	96.0
	2	*C. uliginosa* M28238	93.6
	1	*Melosira*-colonizing bacterium strain IC166	97.3
	1	*Flavobacterium salegenes* M92279	92.7
Cytophaga johnsonae	1	*Flavobacterium columnare* M58781	96.2
High GC, gram positive	1 (0.7)		
Arthrobacter	1	*Micrococcus luteus* M38242	99.3
α-*Proteobacteria*	11 (7.6)		
Roseobacter	8		
Group A	7	*R. algicola*	94.0
Group B	1	Unidentified alpha proteobacterium strain AF022392	95.7
Sphingomonas	3	*Sphingomonas* sp. U85838	93.5
ε-*Proteobacteria*	2 (1.4)		
Arcobacter	2	*A. nitrofigilis* L14627	98.6
γ-*Proteobacteria*	122 (84.1)		
Pseudoalteromonas	29	*P. atlantica* X82134	99.7
Vibrio	15	*V. splendidus* Z31659	99.0
Alteromonas	18		
Group A	10	*A. macleodii* X82145	97.8
Group B	8	*Colwellia psychroerythrus* AB011364	94.5
Oceanospirillum	9	*O. commune* ATCC 27118	95.7
NOR1	2	Unculturable Mariana eubacterium clone D87345	98.6
NOR2	31		
Group A	22	Facultative barophile strain CNTP3 U91588	95.6
Group B	9	*Pseudoalteromonas haloplanktis* D11172	92.9
NOR3	2	Unidentified gamma proteobacterium clone AB004573	94.0
NOR4	1	Clone of aggregate 47 L10949	87.0
Marinobacter	4	*Marinobacter* sp. strain PCOB-2 AJ000647	98.1
Halomonas	9	*H. variabilis* U85873	98.2
Photobacterium	1	*Photobacterium* sp. strain SS9 U91586	95.2
Shewanella	1	*S. putrefaciens* U91593	96.1

[a] Obtained in September and November 1997 and February and August 1998.
[b] In the ARB database.
[c] *Cytophaga-Flavobacterium* cluster.

vored *Oceanospirillum* spp. (five out of nine isolates) and *Arcobacter* spp. (two out of two). Inoculations with unfiltered water and water prefiltered with a 5-μm-cutoff filter predominantly resulted in isolation of strains related to *Roseobacter* spp. (6 out of 8), *Sphingomonas* spp. (2 out of 3), *Vibrio* spp. (15 out of 15), *Pseudoalteromonas* spp. (24 out of 29), *Alteromonas* spp. (18 out of 18), and the gamma-proteobacterial cluster NOR2 (23 out of 31). No preference concerning variations in cultivation conditions (pH, salinity, monomers or polymers, availability of vitamins and trace elements) was observed, except for *Roseobacter*, which was isolated only at pH 8.3 and 35‰ salinity. Attempts to increase the cultivation of strains affiliated with *Cytophaga-Flavobacterium* by using a mixture of polymers as the substrate were unsuccessful. The diversity of the isolates was further evaluated by ARDRA and rarefaction analysis (Fig. 1) as described by Ravenschlag et al. (43). From a total of 145 isolates, 32 *Hae*III patterns were distinguished. The 122 gamma proteobacteria grouped into 21 different patterns (Fig. 1). Rarefaction indicated saturation of the number of ARDRA patterns within this group, but not for all of the isolates (Fig. 1).

16S rDNA clones. Fifty-four 16S rDNA clones were randomly selected for sequencing and phylogenetic analysis. Sequences belonged to several clusters: alpha-proteobacterial cluster SAR116, described by Mullins et al. (35), from Sargasso Sea samples; *Roseobacter* spp.; four gamma-proteobacterial lineages; and the epsilon-proteobacterial genus *Arcobacter*. The most frequent sequences in the clone library (44 of 53) were affiliated with the SAR86 cluster of gamma proteobacteria, first described by Mullins et al. (35) and Fuhrman et al. (16), from the Atlantic and Pacific Oceans, respectively (Table

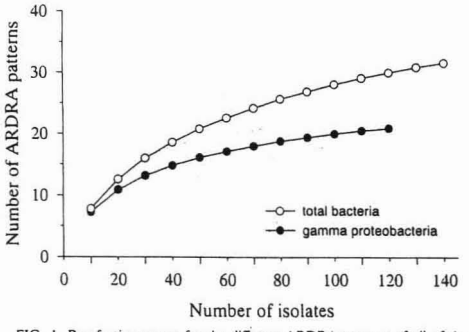

FIG. 1. Rarefaction curves for the different ARDRA patterns of all of the isolates used in this study. The expected number of ARDRA patterns is plotted versus the number of isolates (○). Rarefaction curves were also calculated for the fraction of gamma *Proteobacteria* (●). The dotted lines represent 95% confidence intervals.

TABLE 3. Frequencies and phylogenetic affiliations of North Sea 16S rRNA clones[a]

Phylogenetic group	No. (%) of clones	Next relative to clone[b]	% 16S rRNA similarity
Bacteria	54 (100)		
α-*Proteobacteria*	4 (7.5)		
SAR116 cluster[c]	3	Unidentified alpha proteobacterium clone OM25[d]	95.5
Roseobacter	1	Marine bacterium strain SFR1[e]	97.9
γ-*Proteobacteria*	49 (92.5)		
SAR86 cluster[c]	44 (83)		
Group A	17	Unidentified gamma proteobacterium clone OM10[d]	97.8–99.2
Group B	27	Unidentified gamma proteobacterium clone OCS5 AF001651[d]	96.9–98.1
Thiomicrospira	2	*Coxiella burnetii* D89795	88.4
Unknown affiliation	2	Clone of macroaggregate clone 44[f]	99.2
Unknown affiliation	1	Unidentified gamma proteobacterium clone OM60[d]	95.3
ε-*Proteobacteria*	1 (2)		
Arcobacter	1	*A. butzleri*	92.2

[a] Obtained in September and November.
[b] In the ARB database.
[c] Reference 35.
[d] Reference 42.
[e] Reference 18.
[f] Reference 12.

3). We did not screen more clones, since the library was apparently biased toward the SAR86 cluster.

Oligonucleotide probe design. Probes were designed for some of the most abundant gamma-proteobacterial sequences obtained from isolates and direct 16S rDNA sequence retrieval. Probes ALT1413 and PSA184 target *Alteromonas* and *Pseudoalteromonas* spp., OCE232 targets *Oceanospirillum* spp., NOR1-56 targets the NOR1 lineage, SAR86-1249 targets the SAR86 cluster, and NOR2-1453 targets all members of the NOR2 clusters (Table 1). All of the probes have at least one strong central mismatch with a nontarget sequence (1.4 to 2.0 weighted mismatches) (37), with the exception of probe OCE232, which has only a weak mismatch (0.2 weighted mismatches) with *Methylomicrobium album* and *M. agile*. Optimized hybridization conditions are given in Table 1. In addition, we adapted for FISH the oligonucleotide probes G V and G Rb designed by Giuliano et al. (18), which are targeted to marine *Vibrio* spp. and to *Roseobacter* and *Rhodobacter* spp., respectively. These two probes encompass all 16 isolates affiliated with *Vibrio* spp. and all eight strains of *Roseobacter* spp.

The target groups of all of the probes are shown in the phylogenetic tree of full 16S rDNA sequences in Fig. 2. This tree was calculated only on the basis of nearly full-length sequences and was corrected by taking into consideration the different results of the various tree reconstruction algorithms. Bifurcations indicate branchings which appeared stable and well separated from neighboring branchings in all cases. Multifurcations indicate tree topologies which could not be significantly resolved based on the available data set.

FISH of plankton samples. For the samplings in September and November 1997, as well as February and August 1998, total bacterioplankton cell numbers and percentages of cells hybridizing with specific probes were determined (Table 4). The total cell numbers in the four samples were between 1.2×10^5 (February 1998) and 1.1×10^6 (September 1997) cells per ml. In a similar manner, the rate of detection by FISH varied during the year. Only 31% of DAPI-stained cells hybridized with the general bacterial probe EUB338 in November 1997. This rate increased to a maximum of 71% in August 1998.

Bacteria hybridizing with group-specific probes CF319a, ALF968, and GAM42a were found to be abundant (Table 4). They made up more than half (54%) of all of the cells in August 1998. The most abundant bacteria detected in February 1998 were the alpha proteobacteria, with a maximum of 25%. In August 1998, members of the *Cytophaga-Flavobacterium* cluster constituted 30% of all of the cells collected and alpha proteobacteria constituted 15%. In September 1997, the percentages of alpha proteobacteria and members of the *Cytophaga-Flavobacteria* cluster were more or less equal at 24 and 25% of the DAPI counts, respectively. Probe G Rb for the alpha-proteobacterial genera *Rhodobacter* and *Roseobacter* hybridized with a significant fraction of the cells detected by probe ALF968. This group was most prominent during August 1998, when it constituted 9% of the total community or 60% of the ALF968 counts. Gamma proteobacteria were detected in a more or less constant fraction of 6 to 9% of the total cells in all of the samples examined. Using genus- and cluster-specific probes, we examined the abundances of readily culturable bacteria of the genera *Vibrio*, *Oceanospirillum*, *Alteromonas*, *Pseudoalteromonas*, and *Sphingomonas* and the NOR1 and NOR2 clusters. These never accounted for more than 1% of the total counts. Interestingly, cells detected by G V, ALT1413, PSA184, NOR1-56, and NOR2-1453 were generally large and frequently associated with small clusters of bacteria or particles. Their strong fluorescence with the probes indicated high rRNA contents of cells (Fig. 3). Members of the as yet uncultured SAR86 cluster were much smaller rods (1 to 2 by 0.5 μm) and were less fluorescent (Fig. 3). They showed a maximum in August 1998, when they represented 10% of the total cell numbers.

DISCUSSION

The cultivation of microorganisms in combination with methods based on the 16S rRNA approach (4) gave insight into the culturable diversity and community structure of North Sea bacterioplankton at various seasons. Numerous isolates were screened and phylogenetically identified, thus assessing culturable diversity. Additional phylotypes were detected by cloning from environmental DNA, and the abundances of isolated bacteria and other phylogenetic groups were determined by FISH.

Culturable bacteria with high in situ abundance. In our study, we found two groups of abundant marine bacteria to be culturable. Members of the genus *Roseobacter*, formerly *Erythrobacter*, have been frequently isolated from marine samples

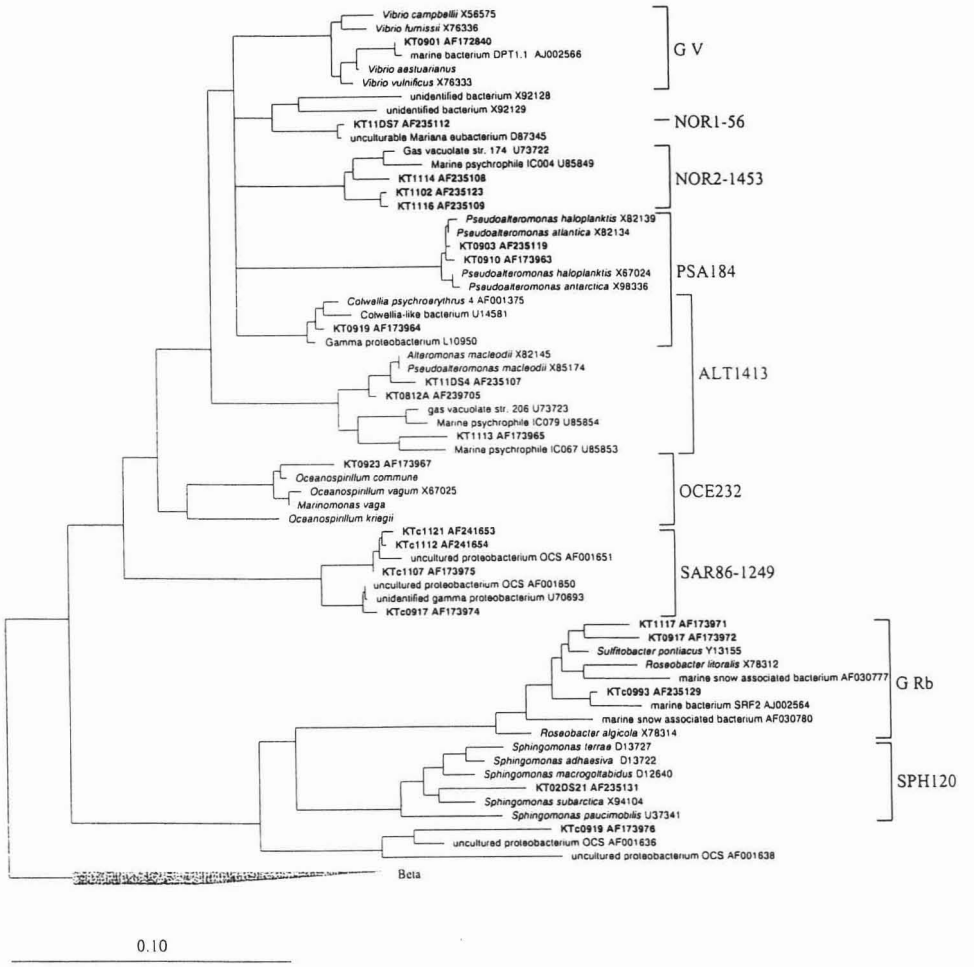

FIG. 2. Phylogenetic tree based on comparative analysis of 16S rDNA from selected clones and isolates of the alpha and gamma subclasses of *Proteobacteria*. Brackets indicate probe specificity. Selected sequences from the beta subclass of *Proteobacteria* were used to root the tree. The bar indicates 10% sequence divergence.

as aerobic, heterotrophic, often pigmented bacteria (50). Sequences related to this genus have also been routinely found in marine clone libraries (16, 18, 35, 42, 55). A study by González and Moran (21) suggested high abundance in coastal seawater of a large phylogenetic branch of marine alpha proteobacteria, including *Roseobacter* spp. In our study, we obtained isolates of *Roseobacter* spp. with oligotrophic medium, found clones related to this group in our North Sea 16S rDNA library, and identified up to 9% of the total counts with probe G Rb. This group was most abundant in the summer, when absolute numbers approach 10^5/ml.

High numbers of members of the *Cytophaga-Flavobacterium* cluster, from which we obtained nine isolates, can be identified by probe CF319a in North Sea bacterioplankton throughout the year. Our failure to retrieve them in the North Sea 16S rDNA library is likely due to a primer bias (20), or the number of clones examined may have been too low. Using other eubacterial primers (28), e.g., 8F and 1492R, *Cytophaga* and relatives have been cloned from macroaggregate samples in the Santa Barbara Channel (12) and from a Bermuda site (16).

Molecular techniques suggest low in situ abundance of frequently isolated bacterial groups. Our extensive cultivation attempts, which included the use of defined artificial medium, enrichments in dilution series (48), and direct plating (60),

TABLE 4. Abundances of various phylogenetic groups in the North Sea as determined by FISH

Date[a] collected	Total cell concn (10^5 ml^{-1})	% Detected by DAPI staining and probe:												
		EUB338	ALF968	GAM42a	CF319a	G Rb	ALT1413	SAR86-1249	G V	OCE232	PSA184	NOR1-56	NOR2-1453	SPH120
11.02.98	1.2	58.9	24.8	5.9	17.5	3.0	<1	<1	<1	<1	<1	<1	<1	<1
20.08.98	8.0	71.4	15.1	9.2	30.3	9.2	<1	10.2	<1	ND[b]	ND	<1	<1	<1
12.09.97	10.5	60.3	23.7	8.0	25.0	4.7	<1	1.7	1.0	<1	<1	<1	<1	<1
13.11.97	3.5	31.2	2.9	6.3	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

[a] The day, month, and year are reported.
[b] ND, not determined.

proved to be highly selective for gamma proteobacteria. Most isolates were closely related to well-known gamma-proteobacterial genera such as *Pseudoalteromonas*, *Alteromonas*, *Vibrio*, and *Oceanospirillum*. We also obtained several gamma-proteobacterial clusters for which we had no close relatives in our 16S rRNA database (NOR1-4; Table 2). FISH with probes targeting the different genera and clusters of culturable gamma proteobacteria never detected more than 1% of the total counts. Except for *Oceanospirillum* spp., the cells detected were large, were attached to particles, and had high cellular rRNA contents. The predominant occurrence of *Alteromonas* and *Pseudoalteromonas* spp. as attached bacteria had been suggested before (1, 12). This was supported not only by our FISH data but also by the lack of isolation of these bacteria from the <1.2-μm fraction.

The genus *Vibrio*, one of the best-known marine taxa, was once claimed to be a major component of the bacterial flora of the sea and to account for nearly 80% of the bacterial community in surface waters of the western Pacific Ocean (52). Likewise, hybridization of community DNA with oligonucleotide probes targeting 16S rDNAs of culturable bacteria suggested the dominance of gamma proteobacteria, in particular, *Vibrio* and *Photobacterium* spp. (44). Yet, these bacteria could not be detected in situ in high numbers in our study and related sequences were not frequent in our 16S rDNA library. Our results thus suggest that these hybridizations to extracted community DNA overestimated the abundances of particular species.

The good growth on agar plates of some gamma proteobacteria is most likely the result of their specific life strategies, which have been studied in detail for *Vibrio* spp. (5, 38, 39). These marine bacteria, which survive carbon starvation for extended periods of time, can grow rapidly at high substrate concentrations with high cellular rRNA contents. It has been shown that upon the onset of carbon starvation a *Vibrio* strain maintains ribosomes for several days in large excess over the apparent demand for protein synthesis (14). The cells of *Vibrio* spp. we detected in situ were all particle attached. Particles are sites of higher nutrient availability, and the large size and high ribosome content of the cells detected could be the result of recent metabolic activity (45). In addition, colonies of *Vibrio* spp. were shiny, which is a characteristic of bacteria producing extracellular slime. Cells with the ability to produce a protective matrix seem to colonize surfaces at the solid-air interface more readily than bacteria that lack this feature (2).

We also isolated three strains of *Sphingomonas* spp. Abundances of 15 to 35% have been reported for this alpha-proteobacterial genus (48). In our North Sea samples, we obtained no FISH counts above the background with SPH120, a 16S rRNA-targeted probe for sphingomonads (36). As in the case of the culturable gamma proteobacteria, FISH data alone are insufficient to decide whether the probe target groups were absent or undetectable due to low rRNA contents (47).

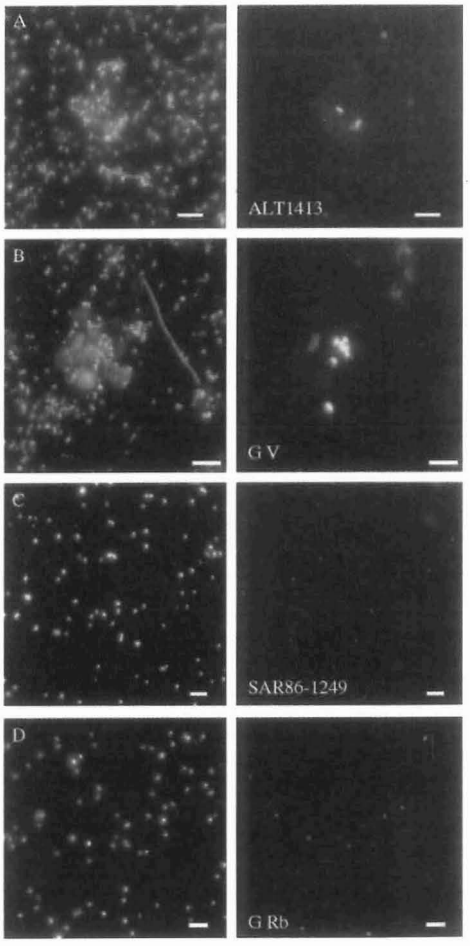

FIG. 3. Epifluorescence micrographs of bacteria in bacterioplankton from the North Sea station Helgoland Roads. Hybridization with CY3-labeled probes (right) and the same microscopic field with UV excitation (DAPI staining, left). Panels: A, probe ALT1413 (18.08.98); B, probe G V (12.09.97); C, probe SAR86-1249 (20.08.98); D, probe G Rb (12.09.97). Scale bars, 5 μm.

Members of the abundant SAR86 cluster remain uncultured. FISH identified up to 10% of the total cells as members of the SAR86 cluster. The small rods were usually not attached to particles, confirming earlier reports that they belong to the free-living fraction of bacterioplankton (1). Sequences related to the SAR86 cluster dominated our North Sea 16S rDNA clone library (Table 3). Nevertheless, no strains affiliated with the SAR86 cluster were among the culturable gamma proteobacteria. Rarefaction analysis of ARDRA patterns (Fig. 1) indicated a low probability of discovering new groups of pelagic gamma proteobacteria by analysis of additional North Sea isolates. After the screening of 70 isolates, 18 ± 3 (average ± 95% confidence interval) ARDRA groups were identified and after the screening of 52 additional isolates, only 3 ± 0.3 new patterns were found. Less than one new ARDRA pattern is predicted for the screening of an additional 20 gamma-proteobacterial isolates. We therefore stopped our efforts to cultivate SAR86.

With regard to the in situ abundance and culturability of heterotrophic marine bacteria, we have evidence for three groups: (i) abundant groups that are culturable, such as *Roseobacter* and members of the *Cytophaga-Flavobacterium* cluster; (ii) abundant bacteria that are still uncultured, such as members of the SAR86 cluster; and (iii) frequently isolated bacteria of the *Vibrio* sp. type with possibly low in situ abundance. Our findings are in obvious contrast to the more optimistic conclusions of Pinhassi et al. and Rehnstam et al. that the most abundant marine bacteria are readily culturable (40, 44).

Although our cultivation attempts failed to isolate new abundant marine pelagic bacteria, the rarefaction analysis of all of our ARDRA patterns (Fig. 1) (not just those from gamma proteobacteria) clearly indicated that further marine bacteria could have been isolated. With high-throughput molecular screening, and early rejection of laboratory weeds, future isolation efforts could be directed to groups such as the alpha proteobacteria and the *Cytophaga-Flavobacterium* cluster. This might provide additional good model organisms of marine aerobic heterotrophic bacteria.

Cultivation strategies. Few new genera of marine bacteria have been cultured since ZoBell's experiments with substrate-amended seawater (23, 40, 55). Using a synthetic medium designed by Schut et al. (48), we may have extended the number of cultured marine species with the strains of our clusters NOR1 to NOR4. Sequences related to the NOR1 cluster have been found in samples from the deep Mariana trench and have been attributed to an unculturable bacterium (30). Applying an appropriate cultivation strategy, we were, however, able to obtain isolates from this phylogenetic lineage. We thus caution against the premature use of the term "unculturable" for bacteria that are only represented by their rDNA sequences in clone libraries.

We are unable to provide the chemical, nutritional, and physical prerequisites for the growth of all of the microorganisms present in natural seawater. Different marine bacteria react differently to confinement (13) and substrate quality and quantity (54). Active metabolism and multiplication might be terminated due to enrichment of toxic products, depletion of essential nutrients (53), and viral infection (58). The growth state of the bacteria at the time of sampling, whether they are active, starved, or dormant, may also strongly influence the success of cultivation.

In principle, for successful enrichments, the physiological requirements of the target microorganism should be known. Most marine bacteria face an oligotrophic environment, but the definitions of the needs of oligocarbophilic microorganisms are as diverse as they are difficult to justify (49). Not even the amount of organic carbon per liter sufficient for growth of oligocarbophilic bacteria is agreed upon, and the appropriate types of carbon sources are in dispute. It is not known if defined mixtures of monomers and polymers, undefined substrates like peptone and yeast extract, or naturally occurring substrates like DMSP (29) and algae lysate will be most suitable for the isolation of hitherto uncultured microorganisms. The quantity and quality of substrates may even play a subordinate role. Our oligotrophic medium, with 1 to 10 mg of C per liter, did not select against *Vibrio* spp. or *Pseudoalteromonas* spp., which also grow well on rich media (40, 60). These bacteria are known to resist nutrient deprivation for long periods of time and to regain active metabolism quite rapidly (5), which is a dilemma for cultivation. Strategies that attempt to prevent substrate-accelerated death (41) by initial incubation at very low substrate concentrations, followed by a gradual increase, will therefore be of little use for the isolation of slowly growing bacteria with potentially long lag phases. The role of phages in the control of CFU is also still unresolved. Bacteriophages of known microorganisms from the North Sea are very host specific and, in general, highly virulent (59). It has been suggested that many bacteria may be apparently unculturable because they are infected by lysogenic viruses (57).

A further problem could be that as yet uncultured bacteria do not form colonies at the air-solid interface. This should not be confused with the general ability to grow on surfaces or submerged particles. Future cultivation attempts could consider (i) filtration (pore size, <1.2 μm) of the inoculum to remove large, highly active, particle-associated bacteria, (ii) dilution to favor dominant bacteria (10), and (iii) colony isolation in semiliquid (soft-agar) medium and subsequent subculturing in liquid medium for bacteria unable to grow at the air-water interface.

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2.

Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts

Heike Eilers, Jakob Pernthaler, and Rudolf Amann

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

HEIKE EILERS, JAKOB PERNTHALER,* AND RUDOLF AMANN
Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen, Germany

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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 culturable 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 culturable genera rather than in the activation of either dormant cells or the original DNA-rich bacterial fraction. *Vibrio* spp. and members of the *Alteromonas/Colwellia* 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 culturable 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 culturable 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) genes (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 culturable bacteria the exception or the rule? In a recent study on North Sea bacterioplankton (14), we found that the most readily culturable 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.

* Corresponding author. Mailing address: Max-Planck-Institut für Marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49 421 2028 940. Fax: 49 421 2028 580. E-mail: jperntha@mpi-bremen.de.

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 culturable 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

TABLE 1. Oligonucleotide probes for in situ hybridization

Probe	Specificity	Probe sequence (5'→3')	Target sites[a] (16S rRNA positions)	% Formamide in buffer	Reference(s)
ALT1413	A/C	TTTGCATCCCACTCCCAT	1413–1430	40	14
SAR86-1249	SAR86 cluster	GGCTTAGCGTCCGTCTG	1249–1265	50	14, 31
G V	*Vibrio*	AGGCCACAACCTCCAAGTAG	841–822	30	20
OCE232	*Oceanospirillum*	AGCTAATCTCACGCAGGC	232–249	40	14
G Rb	*Rhodobacter/Roseobacter*	GTCAGTATCGAGCCAGTGAG	645–626	30	20

[a] *Escherichia coli* numbering (9).

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-prerinsed 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 unfiltered 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 GTTP2500; 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 staining with Hoechst 33342 and SYPRO (Molecular Probes, Eugene, Oreg.) was performed by flow cytometry on a FACStar Plus flow cytometer as described (Becton Dickinson, Mountain View, Calif.) (48). At least 2,000 Hoechst 33342-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; type GTTP2500; Sartorius AG, Göttingen, Germany). Half of the prefiltered 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 (S−) or amended (S+) with a mix of monomers (alanine, L-aspartate, DL-leucine, L-glutamate, L-ornithine, and DL-serine [1 μM]; glucose, fructose, galactose, glycolate, succinate, and mannitol [10 μM]; acetate, lactate, ethanol, and glycerol [15 μM]). At the beginning of the experiment and after 20 and 43 h, 10-ml aliquots were fixed for FISH, and 2-ml aliquots were fixed for flow cytometry (see above).

Batch cultures. For starvation experiments 150-ml triplicate samples inoculated with either *Alteromonas* sp. isolate KT1113 (AF173965), *Oceanospirillum* sp. isolate KT0923 (AF173967), or *Vibrio* sp. isolate KT0901 (AF172840) (14) were incubated at the in situ temperature (16°C) on a rotation shaker (100 rpm) in synthetic seawater (14) to which trace elements, vitamins, and the mix of monomers used for the field incubation were added. At four time points within a period of 50 days, 1.5-ml aliquots were fixed for FISH, immobilized on polycarbonate filters (diameter, 47 mm; pore size, 0.22 μm; type GTTP2500; Millipore, Eschborn, Germany).

FISH. Cells on filter sections were hybridized with group-specific oligonucleotide probes EUB338 (3), ALF968 (20% formamide) (32), GAM42a (30), and CF319a (29). In addition, probes for subgroups of α- and γ-proteobacteria (Table 1) were used. Counterstaining with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) and mounting for microscopic evaluation were performed as described previously (3, 21).

RESULTS

Changes in community composition. During a 43-h enrichment, total cell number increased from $1.6(\pm 0.2) \times 10^6$ (mean ± standard deviation, $n = 12$) cells per ml by a factor of about 2 in the unamended (S−) and about 2.5-fold in the substrate-amended (S+) samples (Fig. 1). This difference between S− and S+ was not statistically significant (Student's t test, $P > 0.05$). After NA addition, no significant changes in cell numbers occurred during the first 20 h in both amended and unamended treatments. Total cell number increased slightly in S−NA+ thereafter. In S+NA+, total cell number almost doubled during the second half of the incubation in spite of the antibiotic. Detection rates of probe EUB338 ranged around 75% ± 12% ($n = 6$) in both 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 $2.1(\pm 0.4) \times 10^5$ ($n = 12$) cells per ml by a factor of 4- to 5-fold and 9- to 10-fold in S− and S+, respectively. This effect was enhanced by incubation with NA. About 13% ± 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 ALT1413, 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. Concomitant with a rise in numbers, the cell volume of these large cells increased even more (Fig. 3). A/C constituted 6 ± 2% 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 increase in absolute numbers in the S+NA− seawater, 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.

Vibrio spp. were enriched more drastically than any other group during incubation, but only in the S+ treatments (Fig. 2). The increase in cell numbers was even stronger in the S+NA+ treatment. At the end of the experiments *Vibrio* spp.

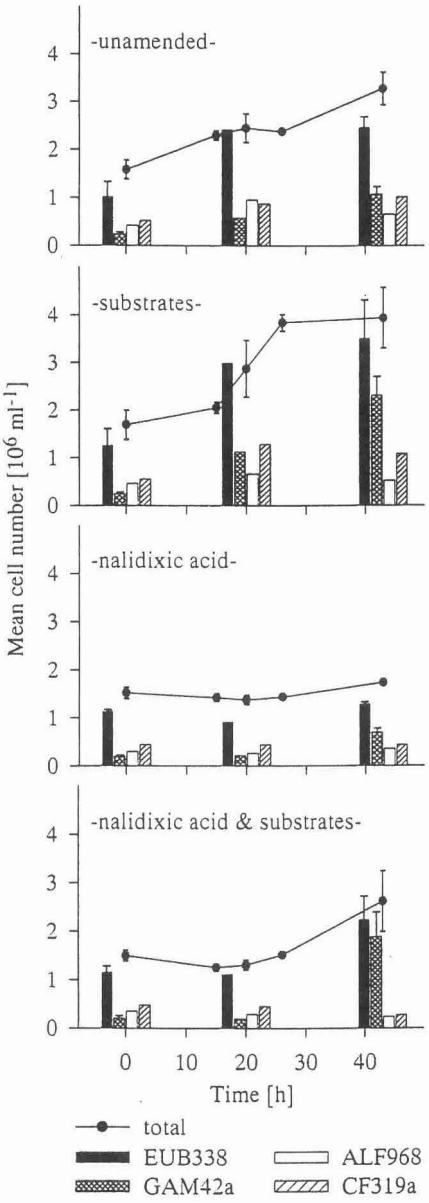

FIG. 1. Mean cell numbers of the total bacterial assemblage (lines) during the enrichment experiments and of cells hybridized with group-specific fluorescent probes (bars). Solid bars, cells stained with probe EUB338; dotted bars, γ-subclass of the *Proteobacteria*; open bars, α-subclass of the *Proteobacteria*; hatched bars, C/F cluster. Error bars indicate standard deviations (n = 3).

constituted 25% ± 1% (g = 6.3 h) and 65% ± 1% of total bacteria in the S+NA− and S+NA+ 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+NA+ treatment.

The morphologically diverse *Rhodobacter/Roseobacter* subgroup 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) (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+NA+ treatments, the fraction of these large cells was much higher (56.0%) than in S+NA− (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 this genus was 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

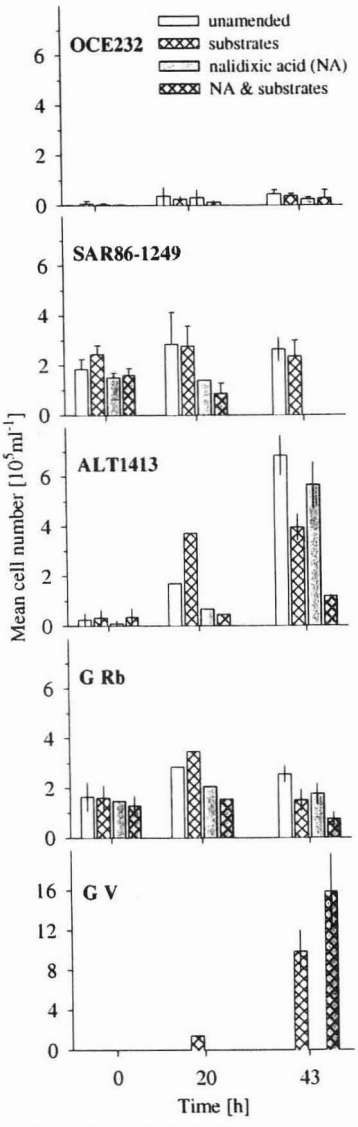

FIG. 2. Mean cell numbers of cells hybridized with probes for various lineages within the γ- and α-proteobacteria in the different treatments. Error bars without caps indicate ranges of replicates; error bars with caps indicate standard deviations of triplicates. Note the different *y* scale in the bottom panel.

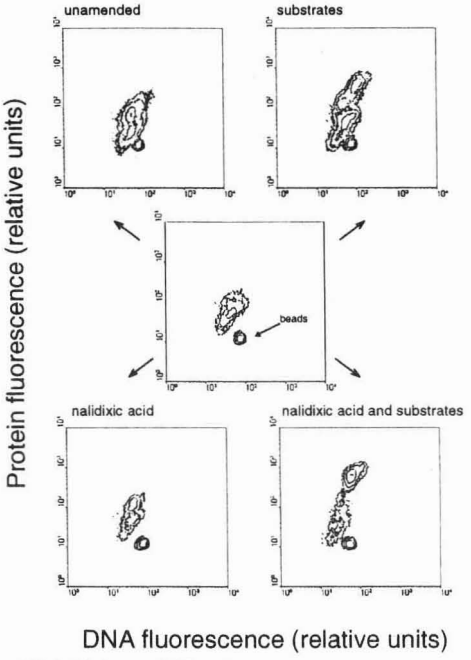

FIG. 3. Relative per cell DNA and protein content (arbitrary units) of the bacterial assemblages at the beginning and end of the various treatments.

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-μm 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 ± 1 standard deviation: unfiltered, $1.52(\pm 0.04) \times 10^6$ cells ml^{-1}, $n = 3$; prefiltered, $1.57(\pm 0.19) \times 10^6$ 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 pe-

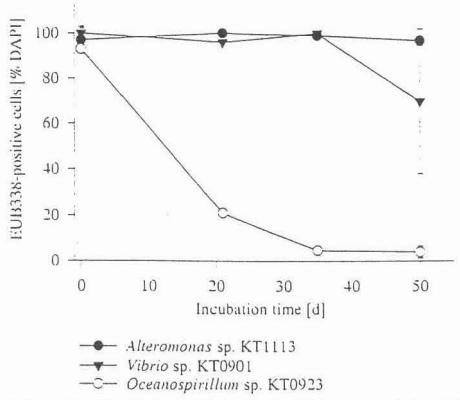

FIG. 4. FISH detection rates of different genera of the gamma subclass of the *Proteobacteria* in stationary-phase batch cultures (probe EUB338, DAPI counterstaining). *Oceanospirillum* sp. KT0923, ○; *Vibrio* sp. KT0901, ▼; *Alteromonas* sp. KT1113, ●.

riod of 24 h (42). γ-Proteobacteria had increased overproportionally already after 20 h of incubation in S−, whereas α-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. γ-Proteobacteria increased from about 15 to 60% and the fraction of C/F again remained constant, but α-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 γ-proteobacteria.

Our study extends previous findings in several respects. We present the response of several individual groups within the marine γ-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 2×10^5 cells ml^{-1}, belonged to the γ-subclass of *Proteobacteria*. Members of a single phylogenetic lineage, the uncultured SAR86 cluster, formed 90% of all γ-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 γ-proteobacterial genera were detected in very low numbers, either attached (*Vibrio* and *Alteromonas*) or free-living (*Oceanospirillum*) in the original North Sea pelagic community (14). In contrast to *Vibrio* and A/C, SAR86 and *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 *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, *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 *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 *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 *Vibrio*, *Alteromonas*, or *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., *Vibrio*, *Alteromonas*, and *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 *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 *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 antibiotic 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-inhibit-

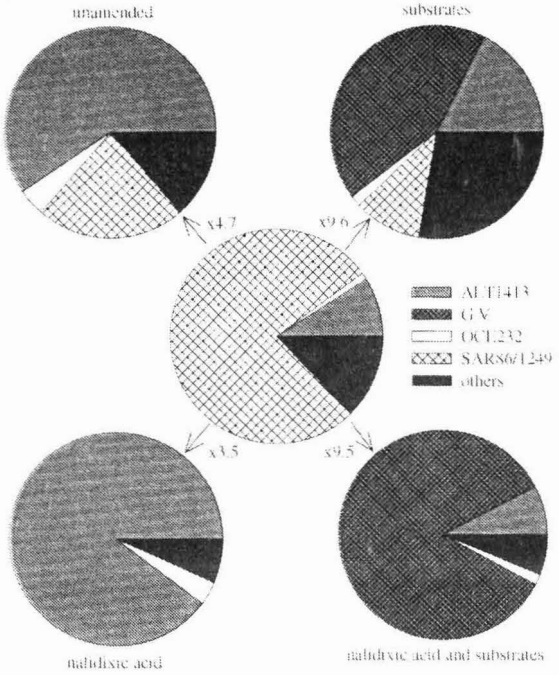

FIG. 5. Percentage of different lineages within the gamma subclass of the *Proteobacteria* at the beginning and at the end of enrichment experiments. Probes: A/C group, ALT1413; *Oceanospirillum*, OCE232; *Vibrio*, G V; SAR86 cluster, SAR86-1249. Factors, increase in γ-proteobacteria compared to the original sample.

ing 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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Seasonal dynamics of cultured and uncultured pelagic bacteria in the North Sea

Heike Eilers, Jakob Pernthaler, Jörg Peplies, Frank Oliver Glöckner, Gunnar Gerdts, Christian Schütt, and Rudolf Amann

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Seasonal Dynamics of Cultured and Uncultured Pelagic Bacteria in the North Sea

HEIKE EILERS,[1] JAKOB PERNTHALER,[1]* JÖRG PEPLIES,[1] FRANK OLIVER GLÖCKNER,[1] GUNNAR GERDTS,[2] CHRISTIAN SCHÜTT,[2] and RUDOLF AMANN[1]

Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen,[1]
Biologische Anstalt Helgoland, D-27498 Helgoland,[2] Germany

Seasonal variations in the community composition of North Sea bacterioplankton were studied by in situ hybridization with rRNA-targeted fluorescently labeled oligonucleotide probes (FISH). During spring and summer the majority of cells affiliated with *Bacteria*. Members of the *Cytophaga/Flavobacterium* cluster (CF) dominated the freeliving microbial community (55%) and formed two distinct maxima in spring and summer. This contrast with the rarity of CF in clone libraries from pelagic marine environments. A culturable lineage of *Cytophaga* sp. constituted about 6% of total cells, but the majority of CF remained unidentified. In early spring, CF was the first phylogenetic group to increase in abundances, followed by the α-*Protetobacteria* and eventually by the γ-*Protetobacteria*. α-*Proteobacteria*, and γ-*Proteobacteria* contributed on average 21 and 15% to the microbial community, respectively. During the period of highest water temperatures and lowest concentrations of inorganic nitrogen (N) and phosphorous (P), a bloom of all three phylogenetic groups coincided with a maximum of the diatom *Lauderia* sp.. Members of the so-called „marine alpha" cluster (25) dominated the α-*Proteobacteria* (67%). However, enrichment and directed cultivation of α-*Proteobacteria* resulted in isolation of bacteria from the "marine alpha" lineage with low in situ abundances. These findings caution against the prevailing view that dominant community members from this lineage are readily culturable. Bacteria from a newly defined phylogenetic cluster with no isolated representatives, NOR5, constitued a large fraction of all pelagic γ-*Proteobacteria* (up to 61%). By reducing concentrations of inorganic N and P in the media to in situ levels and using modified colony transfer, a member of this lineage could be obtained in culture. A variety of isolation strategies might, therefore, be required for the eventual isolation of dominant heterotrophic marine bacteria from different phylogenetic lineages.

INTRODUCTION

In the beginning of marine microbiology morphological descriptions and the Gram staining indicated that a high percentage of abundant pelagic bacteria were recovered in culture collections (60). However, the culturable bacterioplankton remained restricted to a few genera (3). More recently the sequence analysis of 16S rDNA clone libraries derived from mixed amplification products provided evidence that marine picoplankton species composition had been misunderstood and that diversity had been largely underestimated (13, 18, 20). After the finding that more than 99% of marine bacteria do not form colonies on plates (17), it was widely accepted that many important pelagic bacteria can not be readily isolated.

Consequently, considerable effort has been put in the improvement of appropriate cultivation approaches. Many attempts of exploring microbial diversity by cultivation rely on the use of complex media with variations in substrate concentrations (5, 6, 24, 25, 34, 43, 45, 55), a strategy which has been introduced more than 50 years ago (60). So far only few new phylogenetic groups have been discovered by this traditional approach (55). Other improvements of cultivation techniques such as dilution culture (10, 52) are adequate to isolate new marine bacteria, but are so demanding in terms of time and effort that they are rarely used. Enrichment on substrates (DMSP, lignin, chitin) or nutrients (P, N) which are assumed to play an important role in the environment (11, 24, 26, 33, 61) has resulted in extensive cultivation and taxonomic analysis of some readily culturable genera (59) or degraders of particular substrates (5), but so far it has not been shown that these well-studied lineages constitute a detecable fraction of pelagic microbial communities.

Molecular biology has provided tools for the analysis of microbial community composition (1). This allows to track the seasonal contribution of culturable and unculturable pelagic bacteria and archaea in aquatic habitats (15, 35, 41), and thus produces ecological criteria to evaluate the success of particular isolation strategies. Presently, detailed information on the seasonal changes in marine bacterioplankton community composition is still very limited. Bacteria culturable on rich complex media (58), such as *Pseudomonas* sp., *Sphingomonas* sp., *Cytophaga* sp. and *Brevundimonas* sp., were found to constitute a large fraction of the microbial community in a Baltic Sea estuary during distinct seasons (42, 43). This indicates that the brackish water picoplankton as determined by genomic DNA probes can be almost completely dominated by culturable bacteria (42). In contrast, typical

culturable bacteria like *Vibrio* spp., *Sphingomonas* spp. and *Alteromonas* spp. were extremely rare in coastal North Sea bacterioplankton analyzed by fluorescence in situ hybridization and in one instance, an uncultured phylogenetic lineage dominated the γ-*Proteobacteria* (16). It was furthermore shown that the probability of obtaining new uncultured members of the γ-*Proteobacteria* with one particular cultivation approach was marginal. It is unclear if these discrepancies are a consequence of the different cultivation methods and molecular approaches, or of the choice of habitats. Within the α-*Proteobacteria*, members of the „marine alpha" cluster (25) have been shown to be abundant in coastal marine waters, and bacteria affiliating with this lineage have been repeatedly isolated (16, 23, 24, 33). It is, however, not known if cultured representatives from this lineage are indeed abundant in situ.

The goal of this study was to explore if particular modifications of cultivation techniques result in the isolation of α- and γ-*Proteobacteria* that are abundant in coastal North Sea bacterioplankton. We then followed the seasonal changes in the abundances of individual culturable populations and bacterial lineages that contained culturable representatives. Concomitantly, we determined the importance of large phylogenetic groups during the course of a year in order to provide a base for the direction of future cultivation attempts.

MATERIALS AND METHODS

Sampling site and fixation. From January to December 1998 surface water was collected weekly at 1 m depth 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. For cultivation seawater was sampled on August 25th 1999 at the same site. Water was stored at 4°C and further processed within approximately 1 h. For fluorescence in situ hybridization (FISH) portions of 100 ml of unfiltered seawater was processed as described in Glöckner et al. (21).

Enrichment and isolation of marine microorganisms. For cultivation, synthetic seawater MPM as described in (16) was modified (MPM-m). The pH was adjusted to 7.5. The concentrations of NH_4Cl and KH_2PO_4 were reduced to 50 μM and 1.5 μM, respectively. Aliquots (100 μl) of unfiltered seawater were directly spread on triplicate petri dishes containing MPM and MPM-m, both amendend with a mix of monomers (5.7 mg C/l) (16)

and 1% (w/v) agar (Difco). One plate containing MPM-m was selected for further isolation of bacteria. After 2, 7, 12, 15, 19, 29, and 36 days all colonies visible under a binocular microscope at 4x magnification were completely removed from the plate. This was carried out by excising the colony and the underlying agar with a sterile spatula. Isolates were then subcultured in corresponding liquid medium. Some isolates were additionally subcultured in basic MPM, and MPM amended with 0.5% peptone (casein tryptic digested) and 0.1% yeast extract (402 mg carbon per litre).

For enrichment of marine α-*Proteobacteria* in liquid cultures, MPM-m was inoculated with unfiltered and filtered (1.2 μm and 0.45 μm) seawater (1:100 - 1:1000), and the dilutions were incubated at 16°C in the dark. Cultivation conditions were varied by addition either of a mixture of monomers (5.7 mg carbon per liter, (16)), or of polymers (chitin, cellulose, xylan and pectin [1 g per liter of each]). The enrichments were continously screened by FISH with the oligonucleotide probe ALF968, specific for the α-*Proteobacteria*, over a period of nine weeks. At high α-proteobacterial abundances in the enrichments, aliquots were plated out on the corresponding medium with 1% (wt/vol) agar. Representatives of all common colony morphotypes were selected from agar plates and subcultured under the same conditions. Only isolates that hybridized with probe ALF968 were selected for the subsequent analysis.

Phylogenetic analysis. Bacterial 16S rRNA primers 8*f* (5´-AGAGTTTGATCMTGGC-3´) and 1542*r* (5´-AAAGGAGGTGATCCA-3´) were used to amplify almost full-length 16S rDNAs from isolates (9) by polymerase chain reaction (PCR) (51). Amplified 16S rRNA genes from isolates were sequenced by Taq Cycle Sequencing and universal 16S rRNA specific primers using an ABI377 (Applied Biosystems, Inc.) sequencer. Sequence data were analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). A phylogenetic tree was reconstructed using neighbor joining, maximum-parsimony and maximum-likelihood analyses. Only sequences at least 90% complete were used for tree construction. Alignment positions at which less then 50% of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA, which may cause mistakes in the tree topology. The 16S rDNA sequences from isolates generated in this study were deposited in GenBank under the accession numbers AY007676 to AY007684 and AF305498).

Cell counts, FISH and oligonucleotide probe design. Total bacterioplankton counts were determined by epifluorescence microscopy and acridine-orange staining (AODC) (30). Screening of isolates and determination of North Sea bacterioplankton community structure was performed by FISH. Cells from a single colony of each isolate were transferred onto teflon-covered microscopic slides and immobilized by air drying. After dehydration and fixation with 50, 80 and 96% ethanol (w/v), cells on slides and on filter sections were hybridized with oligonucleotide probes EUB338 (2), ALF968 (39), GAM42a (37) and CF319a (36). Counter-staining with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) and mounting for microscopic evaluation were performed as described previously (2, 21).

The oligonucleotide probes NOR5-730, NOR5-130, CYT1448, CYT1438, ROS537, KT13-231, KT09a, KT09b, RC1031, RC1239, Ros7-1029, Erythro69, Rhizo218 (Tab. 1) were designed using the ARB software package. Their specificity was evaluated with the PROBE_MATCH tool of the ARB package against the rRNA database of the Technical University Munich (release 12/98) and by BLAST queueing system of NCBI (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). CY3-labeled probes were synthesized by Interactiva (Ulm, Germany). Stringent hybridization conditions for the newly designed probes were determined on isolates by varying concentrations of formamide (40).

TABLE 1. Oligonucleotide probes used for FISH

Probe	Specifity	Probe sequence (5´-3´)	Targe site[a] (16S rRNA positions	% FA[b]
CYT1448	*Cytophaga* spp.	CTAGGCCGCTCCTTACGG	1448-1465	30
CYT1438	*Cytophaga* spp.	CCGCTCCTTACGGTGACG	1438-1455	30
NOR5-730	NOR5 cluster	TCGAGCCAGGAGGCCGCC	730-747	30
NOR5-130	NOR5 isolate KT71	CCCCACTACTGGATAGAT	130-147	20
ROS537	Marine alpha cluster (25)	CAACGCTAACCCCCTCC	537-553	35
KT13-231	KT1117	ATCTAATCAAACGCGGGCC	231-249	30
KT09b	KT0917	GCTATTCCGTAGATCTGG	136-153	20
KT09c	KT0917	ACTGTGTCCCCTAAAGGA	1025-1042	20
RC1031	KT0202a	ACCTGTCACTATGTCCCG	1031-1049	20
RC1239	KT0202a	TAACTCACTGTAGTTGCCAT	1239-1279	20
Ros7-1029	JP7.1 group	CTGTCACTTGGTCTCTTG	1029-1046	35
Erythro69	JP13.1 group	GCCACTCACCCCGAGGGT	69-106	20
Rhizo218	JP66.1 group	GGGCCCATCCTACTCCGA	218-235	20

[a] *E. coli* numbering (7).
[b] Percent (vol/vol) formamide (FA) in FISH buffer.

RESULTS

Colony forming bacteria. During 37 days of incubation 25±11 (mean ±1 standard deviation) colonies appeared on the basic MPM medium, and 127±1 colonies on the modified one (Fig. 1A). 41% of the isolates that appeared during the first two days of incubation affiliated with the γ-*Proteobacteria* (14 out of 34), 21% with the *Cytophaga-Flavobacterium* branch (CF) of the *Cytophaga-Flavobacterium-Bacteroides* phylum (7 out of 34) and only 9% with α-*Proteobacteria* (3 out of 36). Within the first 15 days the number of additional α-*Proteobacteria* increased 10-fold, whereas the number of additional isolates from the γ-*Proteobacteria* and CF increased by <3-fold. During the second half of the incubation period all additional isolates were α-*Proteobacteria*. Altogether 19 colonies were not transferable. At the end of incubation γ-*Proteobacteria* constituted 29%, CF 12%, α-*Proteobacteria* 33% of the culture collection (Fig. 1B). The number of α-*Proteobacteria* that affiliated with the "marine alpha" lineage (i.e. hybridized with probe ROS537) increased from one single colony during the first two days to a total of 11 strains .

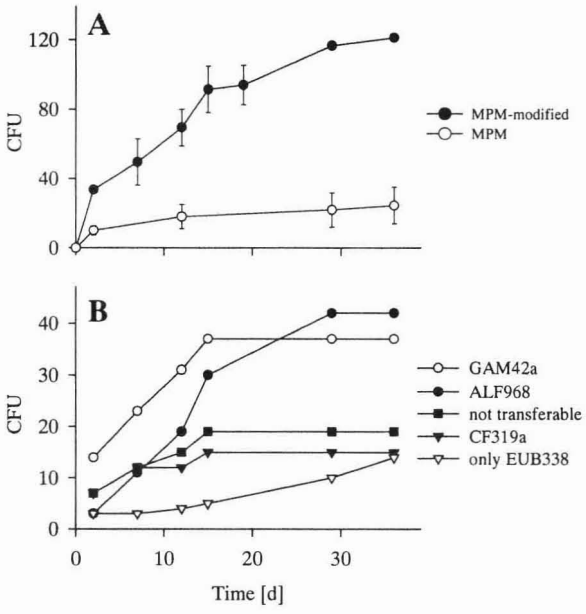

FIG.1. (A) Number of colony forming units per plate (CFU) of North Sea bacteria on artificial seawater medium (MPM, ○), and modified (MPM-modified, •). (B) Phylogenetic affiliation of colonies obtained from MPM-modified screened by fluorescence in situ hybridization (FISH).

16S rDNA sequence analysis of some isolates that did not hybridize with the three group-specific probes revealed that they belonged to other lineages such as the actinobacterial genus *Microbacterium* or to the γ-proteobacterial genus *Colwellia*. During the first two days of incubation well-known α-*Proteobacteria* like *Roseobacter*, γ-proteobacteria like *Pseudoalteromonas*, *Alteromonas*, *Colwellia*, *Photobacterium*, and isolates of the CF

TAB. 2. Frequency and phylogenetic affiliation of North Sea isolates [a]

Phylogenetic group	Representative strain	No. of isolates	Next relative to the isolates [b]	% 16S rRNA similarity	Specific probe
Cyt/Fla [c]					
Cytophaga	KT11ds2 AF(235111)	[d]	*Cytophaga marinoflava* M58770	98.0	CYT1448/CYT1348
	KT0803 (AF235117)		*C. uliginosa* M28238	93.6	
	KT02ds18 (AF235126)		*Flavobacterium salegenes* M92279	92.7	
α-*Proteobacteria*					
"Marine alpha" cluster (25)					ROS537
Group A	JP88.1 (AY007684)	1	Alpha proteobacterium strain 303 (AF022392)	99.7	
Group B	JP7.1 (AY007679)	2	Marine isolate (L15345)	93.3	Ros7-1029
Sulfitobacter	KT0917 (AF173971)	[d]	*Sulfitobacter pontiacus* (Y13155)	94-96	KT09a/KT09b
	KT1117 (AF1117)				KT13-231
	KT0202a (AF305498)				RC1031/RC1239
Unknown affiliation	JP22.1 (AY007682)	2	Marine isolate (Y10914)	98.9	
Rhodospirillum	JP57 (AY007683)	2	*R. salexigenes* (D14431)	88.9	
Sphingomonas	JP63.1 (AY007681)	1	Marine isolate (U85838)	97.9	
Caulobacter	JP68 (AY007678)	1	*C. maris* (AB008850)	96.3	
Erythrobacter	JP13.1 (AY007680)	11	*E. litoralis* (ABO13354)	97.3	Erythro69
Phyllobacterium	JP66.1 (AY007677)	3	*P. myrsinacearum* (D12789)	93.2	Rhizo218
γ-*Proteobacteria*					
NOR5	KT71 (AY007676)	1	γ-proteobacterium KTc1119 (AF235120)	94.7	NOR5-730/NOR5-130

[a] Obtained in August 1999
[b] In the ARB database
[c] *Cytophaga-Flavobacterium* cluster
[d] obtained by (16)

cluster belonging to genera like *Cytophaga*, *Polaribacter* and *Flavobacterium*, were obtained. Representatives of these genera plus representatives of additional genera like *Erythromicrobium*, *Agrobacterium*, *Paracoccus* and *Sulfitobacter* of the alpha class of *Proteobacteria*, *Marinomonas*, *Shewanella* and a well as unknown phylogenetic lineage (NOR6, *Sedimentobacter*, *Alcanivorax*) of the gamma class of *Proteobacteria*, and *Flexibacter* of the Bacteroidetes phylum were obtained within seven days of incubation. Isolates related to NOR1 (16) and an isolate affiliating with a newly defined cluster within the γ-*Proteobacteria*, NOR5, only appeared after 12 days of incubation.

A total of 77 strains was isolated from enrichments containing 21% of α-*Proteobacteria*. 23 strains (30%) hybridized with probe ALF968. For initial phylogenetic analysis partial 16S rDNA sequences of these isolates were determined. Sequences fell into 8 distinct groups within the α-*Proteobacteria* and at least one nearly complete 16S rDNA sequence from each group was obtained. The most frequent isolates were related to the genus *Erythrobacter* (11 out of 23). Five sequences affiliated with three lineages within the "marine alpha" cluster (25), which is a monophyletic group including the genus *Roseobacter* and relatives. The phylognetic affiliation of all analyzed strains is shown in Table 2.

Seasonal community dynamics of marine bacterioplankton as monitored by FISH. The development of total cell counts from North Sea samples in 1998 could be roughly divided into four seasonal phases (Fig. 2A). In winter (January to March) bacterial cell densities ranged around 1.2×10^5 ml^{-1} and remained more or less constant. Cell numbers increased by more than 10-fold during the next three month to a spring maximum of 1.3×10^6 cells ml^{-1}. An in-between period with lower total abundances (0.65×10^6 cells ml^{-1}) divided the spring maximum from a summer maximum. During the summer phase (end of June to begin of September) bacterial abundances reached an annual maximum of 2.1×10^6 cells ml^{-1}. The subsequent decrease of bacterial cell numbers continued during autumn (middle of September to December) to a minimum of 0.7×10^6 cells ml^{-1}.

Detection rate with probe EUB338, targeted to most *Bacteria*, followed the patterns of total abundances (Fig. 2A). In autumn and winter less than 50 to 55% of all cells were detectable by FISH. During this period, on average 10% of total counts affiliated with CF, 10% with the α-*Proteobacteria* and 6% with the γ-*Proteobacteria*. Between 50 and 90% of DAPI-stained particles were visualized by FISH during spring and summer, except during a period of distinctively lower total cell counts (June-July). During spring and summer,

virtually all cells detectable by EUB338 could be assigned to CF and the α- and γ-*Proteobacteria* with the oligonucleotide probes CF319a, ALF968, and GAM42a, respectively. On average, 33% of cells affiliated with CF, 20% with the α-proteobacteria and 14% with the γ-*Proteobacteria*.

Members of CF increased in relative community contribution both during spring and during summer, and reached maximal abundances of 0.45×10^6 cells ml^{-1} and 1.2×10^6 cells ml^{-1}, respectively (Fig. 2B). They constituted up to 60% of all pelagic bacteria between April and July, whereas their relative abundances remained low (up to 20% of total) in autumn and winter. Bacteria affiliating with culturable members within the *Cytophaga* group (16)(Fig. 2C, Fig. 3C) were detectable between April and September, and constitued a constant fraction of 6±2%. This corresponded to a spring and summer maximum of 0.75×10^5 cells ml^{-1} in May and 1.1×10^5 cells ml^{-1} in July/August.

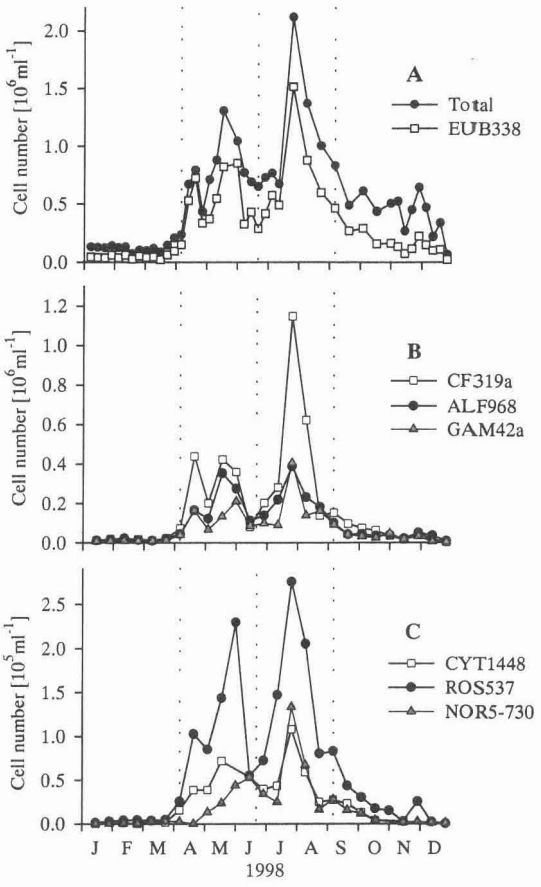

FIG. 2. (A) Seasonal fluctuations in the absolute abundances of total cells and *Bacteria;* (B) of *Cytophaga/ Flavobacterium*, α-*Proteobacteria* and γ-*Proteobacteria*; (C) of *Cytophaga* spp., the "marine alpha" cluster, and the NOR5 cluster in the North Sea bacterioplankton at Helgoland Roads in 1998. Note the different y-scales.

The relative abundances of bacteria detected by ALF968 ranged between 15 to 33% of total counts during spring and summer. α-*Proteobacteria* formed a later spring maximum than CF in mid May (0.35×10^6 cells ml^{-1})(Fig. 2B). In contrast with CF, the absolute abundances of α-*Proteobacteria* were not higher during the second summer maximum (July/August). *Roseobacter* spp. and other members of the "marine alpha" cluster

(25), as detected by probe ROS537 (Fig. 3A), formed the majority of the α-*Proteobacteria* between April and September (mean, 67%; range, 44-100%)(Fig. 2C). In autumn and winter, when α-*Proteobacteria* constituted 10 to 20%, members of the marine alpha cluster were only of minor importance. Several culturable representatives from various phylogenetic lineages within the "marine alpha" cluster (isolates KT0202a, KT0917, KT1117, JP7.1, Tab.

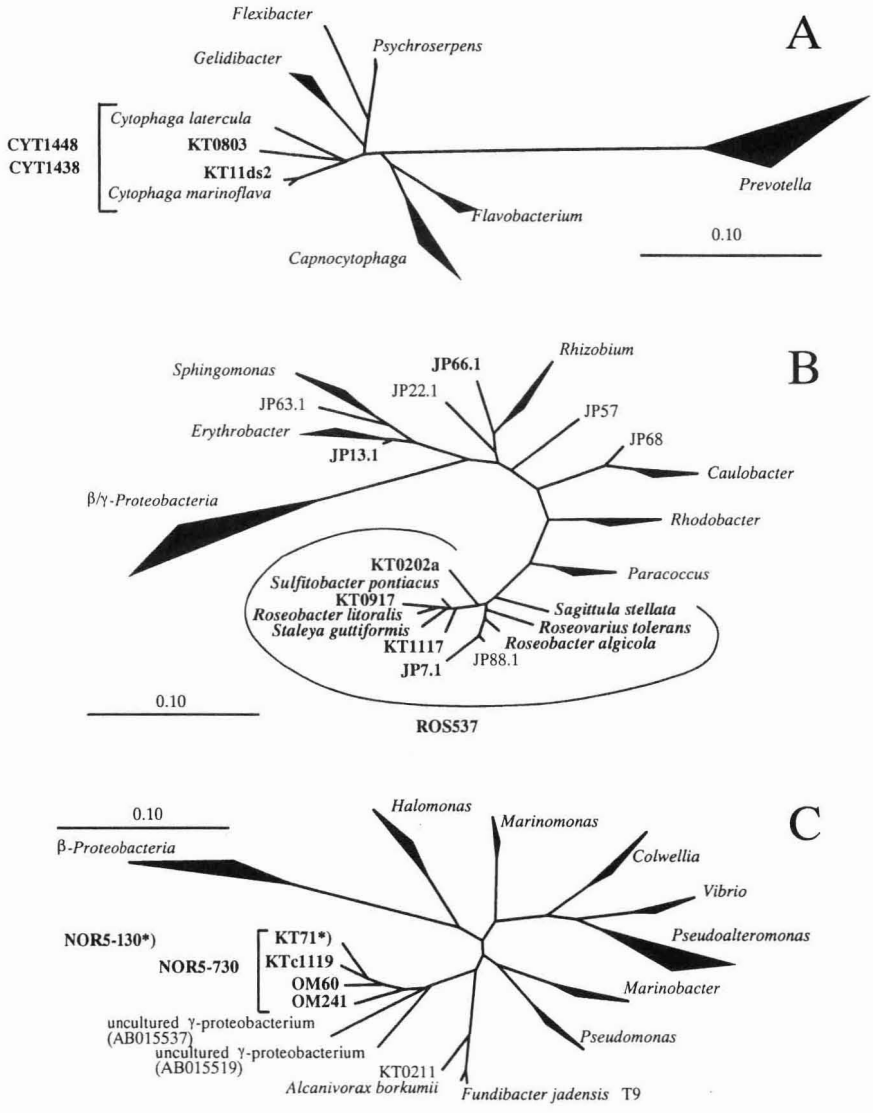

FIG. 3. Phylogenetic trees based on comparative analysis of 16S rDNA from selected isolates of (A) the *Cytophaga/Flavobacterium* cluster, (B) α-*Proteobacteria*, and (C) γ-*Proteobacteria*. Brackets indicate probe specifity. The bar indicates 10% sequence divergence.

2) and from other α-proteobacterial lineages (isolates JP13.1, JP66.1, Tab. 2) formed populations ≤ 1% of all cells in situ, as determined by FISH with specific probes (Tab. 1).

The fraction of bacteria hybridizing with probe GAM42a reached its first maximum ($0.2\pm\times10^6$ cells ml^{-1}) at the end of spring (June) (Fig. 2B). On average γ-*Proteobacteria* were less abundant than both α-*Proteobacteria* and CF, but increased to similar densities as the α-*Proteobacteria* during summer (0.4×10^6 cells ml^{-1}). Relative abundances of γ-*Proteobacteria* remained below 10% of total bacteria in autumn and winter, and fluctuated between 10 and > 20% in spring and summer. During a period of about three months (May-August) members of the NOR5 cluster, as detected by probe NOR5-730, formed a large fraction (up to 61%) of γ-*Proteobacteria*. They reached a maximum of 6-8% of total counts between June and July, respectively, which corresponded to abundances of 0.5×10^5 cells ml^{-1} in June and 1.35×10^5 cells ml^{-1} in July/August (Fig. 2C).

DISCUSSION

General changes of community structure. Our study shows that members of the CF cluster clearly dominated the bacterioplankton community in spring and summer both in absolute and relative abundances (Fig 2B). Moreover, CF formed two very distint peaks of relative community contribution, whereas the relative abundances of α- and γ-*Proteobacteria* showed less significant changes during the year. This is evidence that CF is a major and dynamic component of the freeliving pelagic microbial community during different seasons, which is in striking contrast with the rarity of sequences related to CF in clone libraries from marine bacterioplankton (22). Our findings moreover question the common opinion that pelagic representatives from this lineage are predominatly particle-attached (14, 48). The overproportional increase of abundances of CF during phytoplankton blooms suggested that algal release of dissolved compounds provided an important carbon source for this bacterial group. Rosselló-Mora et al. (50) showed that the addition of complex organic material promoted the growth of CF in marine sediments.

Two subsequent blooms of phytoplankton in the German Bay during spring (April-May) and summer (June-August) (28) seemed to be promoters of major microbial community transitions (Fig. 2B). This is easily conceivable considering the close relationship between

transitions (Fig. 2B). This is easily conceivable considering the close relationship between these two compartments, e.g. the flux of organic matter (4). During spring the shifts in relative abundances of different phylogenetic groups of pelagic bacteria appeared to reflect the succession of dominant diatom species (28). In the beginning of April, a population of *Coscinodiscus* sp. formed, followed by a bloom of *Rhizosolenia* spp. in May (data not shown), a development which coincided with a community transition from CF to α-*Proteobacteria*. Similar changes of a spring microbial community structure was reported from brackish Baltic Sea waters (42). During an artificially induced diatom bloom in a mesocosm enhanced growth rates and enzyme activities were described mainly within the α-*Proteobacteria* and the *Cytophagales* (49). In contrast to these studies, we also observed a distinct increase of γ-*Proteobacteria* at the end of May, when the diatom *Lauderia* sp. dominated phytoplankton biomass (28).

During the second part of the spring bloom a population of cells not detectable by the bacterial probe EUB338 cells was developing, and remained detectable in spite of decreasing cell numbers between May and beginning of June (Fig. 2A). We hypothesize that a fraction of these cells might have been *Archaea*, and presently we are investigating the seasonal successions of pelagic archaea using oligo- and polynucleotide probes (29). In Antarctic coastal water archaeal rRNA and phytoplankton densities were significantly negatively correlated (38). Alternatively, some phylogenetic lineages of *Bacteria* are not detected by probe EUB338, such as the *Planctomycetes* or *Verrucomicrobia* (12). In a river ecosystem, a maximum of planctomycetes occurred when water temperature was high and chlorophyll *a* concentration was low (8). Since the sum of specifically hybridized cells did not add up to the number of all FISH-detectable cells both in June and during autumn and winter, we assume that representatives of other groups than of the three studied lineages were also present in North Sea plankton. For example, *Actinobacteria* (46), β-*Proteobacteria* , and members of the *Fibrobacter/Chlorobium* phyla (SAR406) (27, 55), were all described from coastal bacterioplankton but have not been investigated in our study.

The summer phytoplankton community in the German Bight in 1998 was mainly composed of diatoms and various phytoflagellates (28). After an in-between period of low total abundances (June), CF were again the first to increase in numbers, followed by members of the α-*Proteobacteria*. All three phylogenetic groups formed their annual maximum at the same time point (July/August), during a distinct bloom of the diatom *Lauderia* sp. CF clearly dominated the summer bacterioplankton, whereas α- and γ-

Proteobacteria each represented about one third of CF abundances. This again differs from the development in brackish Baltic Sea waters, where α-*Proteobacteria* were the most important members of the summer community (42).

The observed decrease of total cell number during the second half of summer is probably the consequence of bacterial mortality due to protists or viruses (19, 44). The most pronounced decline, both in relative (55 to 15%) and absolute (1.2 to 0.2×10^6 cells/ml) amounts, was observed within CF, whereas α- and γ-*Proteobacteria* were less affected in their relative community contribution. This indicates that bacteria from individual phylogenetic groups may show different sensitivity to various mortality factors (57). During autumn and winter total picoplankton abundances continuously decreased. The low detection rates with probe EUB338 during this periods is probably the consequence of reduced growth (31).

Dominant members of the "marine alpha" cluster remain uncultured. In coastal North Sea bacterioplankton members of the genus *Roseobacter* and of other genera grouped within the "marine alpha" lineage (25) constituted the majority of α-*Proteobacteria* (Fig. 2C). Some isolated representatives of this cluster have been identified as dimethylsulfoniopropionate (DMSP) degrading bacteria (24, 33). Directed cultivation from enrichments and reduction of nutrients in agar plates both resulted in an increase of numbers of isolated marine α-*Proteobacteria*, which constituted 30% of all selected colonies rather than 7.6% as reported previously (16). However, several isolates that were closely related to culturable bacteria within the "marine alpha" cluster (25)(Tab. 2) were not detectable in the environment by FISH with specific probes during various seasons. Two isolates obtained during a previous study (16) (KT0917 and KT0212a) ranged at the lower FISH detection limit of 1% during August (data not shown). In addition, other α-*Proteobacteria* isolated from enrichments which do not belong to the "marine alpha" cluster , such as *Erythromicrobium* sp. and *Rhizobium* sp. (Tab. 2), were below FISH detection limit. This agrees with our earlier findings that abundant community members may resist cultivation (16). Our results specifically suggest that many readily culturable members of the "marine alpha" lineage are probably not common in situ.

Novel numerically abundant culturable γ-*Proteobacteria*. γ-*Proteobacteria* formed one distinct bloom during summer (Fig.2B). During this time temperatures increase from 10 to 18°C, and the concentration of inorganic nitrogen and phosphorous were minimal

(<10 μmol N/l, 0.5 μmol P/l) (28). Bacteria affiliating with the newly defined NOR5 cluster (Fig. 2C) constituted a major fraction of γ-*Proteobacteria* during two blooms of the diatom *Lauderia* sp. in May/June (61%) and July/August (33%). Previously described members of this lineage are known exclusively from 16S rDNA sequences obtained by PCR-based cloning from coastal marine samples (47). Here, a representative of this cluster (strain KT71) could be isolated after a substantial reduction of P and N in the cultivation medium to the ambient environmental concentrations of these nutrients during summer. Using an oligonucleotide probe targeted only to the 16S rDNA sequence of this isolate (NOR5-130, Tab. 1) bacteria closely related to this strain could subsequently be detected in the environment at similar densities as with probe NOR5-730 (Fig 2C). Eilers et al. showed that the probability to isolate new γ-*Proteobacteria* on the original MPM medium was marginal irrespective of a variety of different carbon sources (16). Therefore, the cultivation of a member of NOR5 was a direct result of the modified isolation strategy. Preliminary observations indicate that KT71 does not grow on a medium containing 0.5% pepton and 0.1% yeast extract, but grows without additional substrates or with low amounts of organic carbon (H. Eilers, unpubl. data). This is a first indication that the NOR5 lineage habours truly oligocarbophilic marine bacteria (53).

Cultivation of abundant marine bacteria. The significant increase in the total number of colony forming units at reduced N and P concentrations (Fig 1A) suggests that there might be an analogy to the concept of substrate-accellerated death (54) of carbon-limited bacteria (32). This finding should caution against cultivation attempts of pelagic marine bacteria on common complex media that potentially contain excessively high concentrations of inorganic nutrients. In addition, the removal of whole colonies successfully prevented the frequently observed overgrowth of slowly growing bacteria by more rapidly developing colonies (56). There was a clear difference in the affiliation of the rapidly colony-forming cells and those bacteria that appeard only after extended incubation period (Fig. 1B). For example, the isolate KT71, affiliated with the new NOR5 cluster, produced a visible colony much later than many other, readily culturable bacteria.

Culturable bacteria affiliating with CF that were obtained during a previous study (16), could be detected by both probes CYT1448 (Fig. 2C) and CYT1348 (data not shown). They constituted a constant fraction of about 5.5% of all bacteria during spring and summer, and were only a minority of all CF. Eilers et al. reported that many bacteria commonly isolated by a particular strategy are rare in the environment (16). Bacteria targeted by

CYT1448 originated from the same culture collection. Therefore, we need to limit our previous conclusions to the γ-*Proteobacteria*. However, the majority of common marine CF are probably not yet isolated. Considering the predominance of CF in coastal North Sea bacterioplankton, increased attention should be paid to the specific isolation of members of this lineage from the pelagic evironment in the future.

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Mikhail V. Zubkov, Bernhard M. Fuchs, Heike Eilers, Peter H. Burkhill, and Rudolf Amann. 1999. Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. Applied and Environmental Microbiology **65**:3251-3257.

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