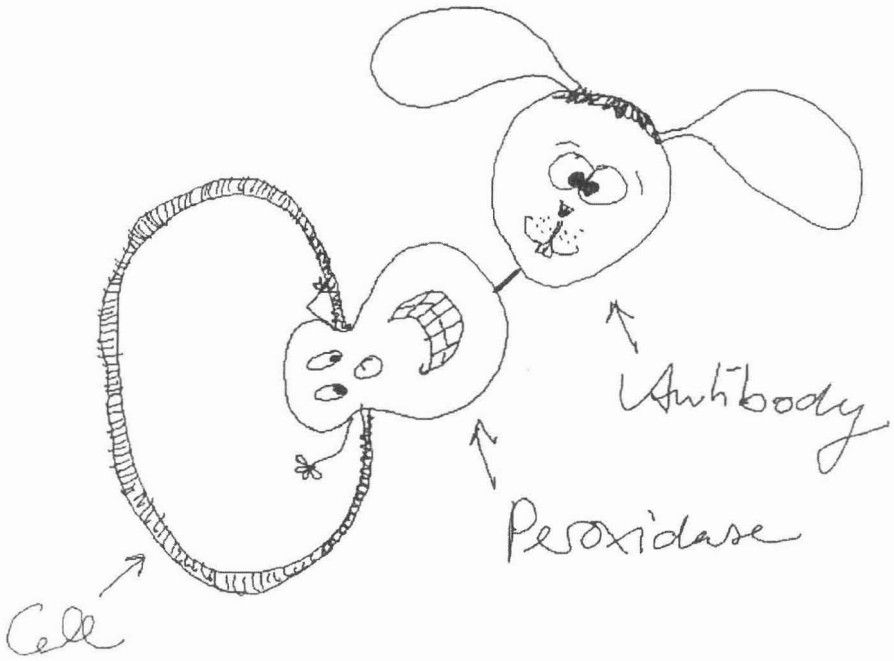
Zytometrische und zytochemische Methoden zur Untersuchung marinen Picoplanktons



Annelie Pernthaler

Zytometrische und zytochemische Methoden zur Untersuchung marinen Picoplanktons

Cytometric and cytochemical methods for the investigation of marine picoplankton

Dissertation

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie der

Universität Bremen

vorgelegt von

Annelie Pernthaler

Februar 2002

Die vorliegende Arbeit wurde in der Zeit von Oktober 1998 bis Februar 2002 am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

1. Gutachter: Prof. Dr. Rudolf Amann
2. Gutachter: Prof. Dr. Ulrich Fischer

Tag des Promotionskolloquiums: 12. April 2002

Inhaltsverzeichnis

Zusammenfassung

Im Rahmen dieser Arbeit wurden Färbemethoden entwickelt, die eine verläßliche Bestimmung von Populationsgrößen mariner Mikroorganismen erlauben und Auskunft über bestimmte Facetten bakterieller Aktivität geben.

Mittels quantitativer Fluoreszenz-*in situ*-Hybridisierung (FISH) mit rRNA-gerichteten Oligonukleotidsonden und konfokaler Laserscanningmikroskopie wurden zelluläre 16S rRNA-Konzentrationen von zwei marinen Isolaten in unterschiedlichen Wachstumsphasen gemessen. Während der frühen stationären Phase zeigten die Stämme deutliche Unterschiede in ihrer rRNA-Konzentration, was auf ihre verschiedenen Wachstumsstrategien hinwies. Der rRNA-Gehalt von Einzelzellen in einer komplexen natürlichen Gemeinschaft ist daher kein einfach interpretierbarer Wachstumsparameter und liefert unter Umständen widersprüchliche Information über die Aktivität verschiedener Bakterienarten.

Der oft geringe Ribosomengehalt langsam wachsender Zellen in oligotrophen marinen Systemen schränkt die Anwendung von FISH mit einfach markierten Oligonukleotidsonden auf bestimmte Habitate ein. Wir untersuchten daher im marinen Plankton systematisch die Detektionsraten und verglichen sie mit einem anderen Ansatz, der FISH mit mehrfach markierten Polyribonukleotidsonden. Die Detektionsraten mit diesen längeren Sonden waren in Winterproben aus der Nordsee und in tieferen Wasserschichten im Pazifik signifikant höher als mit einfach fluoreszenzmarkierten Oligonukleotiden. Im Oberflächenwasser der Deutschen Bucht wurde mit Polynukleotidsonden eine Blüte von *Euryarchaeota* nachgewiesen. Marine Archäen bildeten während der Frühlings- und Sommermonate 1998 bis zu 30% des heterotrophen Picoplanktons.

Ein Protokoll für FISH von Nordseebakterien mit enzymmarkierten Oligonukleotidsonden und Tyramid-Signalverstärkung wurde entwickelt. Die Sensitivität der neuen Methode war vergleichbar mit FISH mit Polyribonukleotidsonden. Es konnten Populationsgößen von Bakterien bestimmt werden, die mit dem bisherigen FISH-Ansatz nicht oder nur sehr eingeschränkt detektierbar waren, z.B. der kosmopolitischen SAR86-Gruppe. Zudem wurde ein weiteres Protokoll für die immunzytochemische Detektion von Bromdeoxyuridin (BrdU) in neu synthetisierter DNA von Bakterienzellen erarbeitet. Mittels der FISH mit enzymmarkierten Oligonukleotidsonden und Antikörperfragmenten gegen BrdU können damit einzelne DNA-synthetisierende Zellen in definierten Populationen des marinen Bakterioplanktons identifiziert werden. Der *in situ*-Nachweis von DNA-Synthese für Zellen der SAR86-Gruppe zeigte, daß die geringe Ribosomendichte und das kleine Zellvolumen bei dieser Gruppe kein Hinweis auf Inaktivität oder Dormanz ist.

Summary

During this study I developed staining methods that allow a more reliable quantification of the population sizes and that address aspects of bacterial activity of marine microorganisms.

Cellular 16S rRNA concentrations of two marine isolates were determined during different growth phases by quantitative fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy. Significant differences of rRNA concentrations between the strains were detected during early stationary phase, and this was in accordance with their contrasting growth strategies. The rRNA content of single cells in complex communities is, therefore, a growth parameter that cannot be easily interpreted and activity estimates from rRNA concentrations might even produce contradictory results on the community level.

Slowly growing bacterial cells with low rRNA content are sometimes not quantitatively detected by whole-cell hybridization with monolabeled fluorescent probes. We studied the limitations of this FISH technique in the marine plankton, and compared it to FISH with multiply labeled polyribonucleotide probes. Detection rates with polynucleotide probes in North Sea winter samples and in deeper water layers in the coastal Pacific Ocean were significantly higher than with oligonucleotide probes. A bloom of *Euryarchaeota* was detected in surface waters of the German Bight. During spring and summer these marine archaea formed up to 30% of picoplankton cells.

A protocol was developed for FISH of marine bacterioplankton with enzyme labeled oligonucleotide probes and tyramide signal amplification. The sensitivity of this method was comparable to FISH with polyribonucleotide probes. Members of the SAR86 clade were not detectable by FISH with directly fluorescently labeled probes, but could be readily quantified by the new approach. Subsequently, another protocol was developed for the immunocytochemical whole-cell detection of bromodeoxyuridine (BrdU) incorporated into newly synthesized DNA. Individual DNA-synthesizing populations in marine bacterioplankton were distinguished by simultaneous FISH and antibody staining, combined with enzymatic signal amplification. In situ DNA synthesis was demonstrated for members of SAR86, indicating that a low ribosome content and a small cell size does not imply dormancy in this group.

Teil I

Darstellung der Ergebnisse im Gesamtzusammenhang

A

Einleitung

Bakterien und Archäen sind wichtige Komponenten mariner Ökosysteme (Pomeroy, 1974; Azam *et al.*, 1983; Fuhrman *et al.*, 1989; Fuhrman *et al.*, 1992; DeLong *et al.*, 1994; Fuhrman and Davis, 1997; DeLong *et al.*, 1999; Glöckner *et al.*, 1999; Murray *et al.*, 1999; Beja *et al.*, 2000; Boetius *et al.*, 2000; Karner *et al.*, 2001). Die Neubewertung der Rolle der Mikroorganismen im Plankton ist eng verknüpft mit der Verbreitung von drei methodischen Weiterentwicklungen: fluoreszenzmikroskopische Zähltechniken zur direkten Abundanzbestimmung (Hobbie *et al.*, 1977; Porter und Feig, 1980) anstelle von Koloniezählungen (Jannasch und Jones, 1959), Inkubation mit radioaktiv markierten Substraten, die eine Einschätzung des Wachstums und der Aktivitäten mikrobieller Gemeinschaften erlauben (Brock, 1967; Fuhrman und Azam, 1982; Simon und Azam, 1989), sowie die Analyse von Nukleinsäuresequenzen zur Untersuchung der mikrobiellen Diversität (DeLong *et al.*, 1989; Giovannoni *et al.*, 1990; Amann *et al.*, 1995). Heute wissen wir, daß ein beträchtlicher Teil der lebenden Biomasse im Meer von Mikroorganismen gebildet wird (in Küstengebieten > 90 % des gesamten partikulären Kohlenstoffs [Karl, 1986]) und etwa die Hälfte des photosynthetisch fixierten Kohlenstoffs durch die sogenannte "mikrobielle Schleife" (microbial loop) umgesetzt wird (Azam *et al.*, 1983; Azam, 1998; Ducklow, 1999). In jüngerer Vergangenheit wurde jedoch auch erkannt, daß ein tieferes Verständnis der Ökologie von mikrobiellen Gemeinschaften auch im marinen Plankton eine präzise und vor allem gleichzeitige Information über die Größe und Aktivität individueller Populationen erfordert (Amann und Kühl, 1998; Cottrell und Kirchman, 2000). Mit Methoden, welche diese beiden Aspekte verbinden, konnte beispielsweise gezeigt werden, daß Bakterien aus der *Cytophaga-Flavobacterium*-Verwandtschaft eine wichtige Rolle beim Abbau von Chitin spielen (Cottrell und Kirchman, 2000), und daß in einer Blüte von coccolithophoren Algen *Roseobacter* spp. maßgeblich am Umsatz von Dimethylsulfopropionat beteiligt war (Zubkov *et al.*, 2001a). Daher konzentriert sich die gegenwärtige Forschung u.a. auf die Verbesserung von Techniken, die auf Einzelzellebene die taxonomische Zuordnung, die Messung verschiedener Aktivitätsparameter, oder beides zugleich ermöglichen (Lee *et al.*, 1999; Ouverney und Fuhrman, 2001; Sherr *et al.*, 2001; Zubkov *et al.*, 2001b).

Die vorliegende Dissertation befaßt sich mit der Entwicklung und dem Vergleich von fluoreszenzmikroskopischen Methoden zur Identifizierung und Aktivitätsbestimmung einzelner Bakterienzellen im heterotrophen Picoplankton.

1. Quantitative Fluoreszenz-*in situ*-Hybridisierung

Mittels Fluoreszenz-*in situ*-Hybridsierung (FISH) mit rRNA gerichteten Oligonukleotidsonden lassen sich selektiv Zellen einer bestimmten phylogenetischen Zugehörigkeit anfärben (DeLong *et al.*, 1989; Amann *et al.*, 1990; Amann *et al.*, 1995; Amann *et al.*, 1997a). Das Hybridisierungssignal von einfach markierten Sonden spiegelt dabei nicht nur die Identität einer Zelle wieder, sondern auch die Menge an zellulärer 16S bzw. 23S rRNA. In *Eschericha coli* konnten die FISH-Signalintensitäten logarithmisch wachsender Zellen sowohl mit dem zellulären rRNA-Gehalt als auch mit Wachtumsraten korreliert werden (Poulsen *et al.*, 1993; Wallner *et al.*, 1993). Eine direkte Beziehung zwischen dem zellulärem rRNA-Gehalt und der Wachstumsrate von *Salmonella typhimurium* während "steady-state" Bedingungen im Chemostaten wurde bereits vor über 40 Jahren von Schaechter et al. (Schaechter *et al.*, 1958) gezeigt. Doch auch wenn eine derartige Korrelation zwischen Wachstumsrate und Ribosomengehalten in Bakterien inzwischen für verschiedene, auch marine Mikroorgansismen bestätigt werden konnte (Kemp *et al.*, 1993; Poulsen *et al.*, 1993), sind solche Erkenntnisse nicht unkritisch auf Umweltmikroorganismen übertragbar (Amann *et al.*, 1995).

Zwei grundsätzliche Schwierigkeiten behindern die einfache Bestimmung der Wachstumsgeschwindigkeit von Mikroorganismen in komplexen Lebensgemeinschaften aus der Intensität von rRNA Hybridisierungssignalen. Erstens muß in Reinkultur dokumentiert werden, daß die untersuchte Bakteriengruppe die „erwartete" Beziehung zwischen rRNA Gehalt und Wachstumsrate aufweist. Das zweite Problem liegt in der Annahme, daß mikrobielle Populationen in Umweltproben sich tatsächlich im "steady-state" Wachstum befinden, denn nur unter solchen Bedingungen ist die obengenannte Beziehung zwischen Ribosomengehalt und Wachstum nachweisbar. In einer kontinuierlichen Kultur bleibt unter Chemostatbedingungen die Synthese aller zellulären Komponenten konstant (Maaloee und Kjeldgaard, 1966), und nur unter solchen Bedingungen reflektiert die Ribosomenkonzentration die Ribosomensyntheserate. Wachsen Zellen hingegen nicht im Gleichgewicht, könnte es sehr große Unterschiede

zwischen der Synthese und der aktuellen Konzentration von Ribosomen geben (Flärdh *et al.*, 1992; Givskov *et al.*, 1994). Dann besteht keine direkte Korrelation zwischen Wachstumsaktivität und der zellulären rRNA Konzentration von Bakterienzellen. Von „steady-state" Bedingungen ist jedoch in einer Umwelt von variablen physikalischen (Temperatur), chemischen (pH, Nährstoffe) und biologischen (Fraßdruck, Konkurrenz) Bedingungen (Molin und Givskov, 1999) nicht auszugehen.

Ein Beispiel für den oft fehlenden Zusammenhang von rRNA Konzentrationen und dem Wachstum von Zellen in einer komplexen Umwelt ist die Aktivität und Verteilung von intestinaler *Escherichia coli* bei Mäusen mit monospezischer Darmflora. In einem solchem System ist es relativ einfach, die mittlere Generationszeit von *E. coli* durch Bestimmung der Abundanz der gesamten Population, des Darmvolumens und der Exkretionsrate zu berechnen. Basierend auf diesen einfachen Zählmethoden, kann die Generationszeit auf etwa 10 – 20 Stunden geschätzt werden (Freter *et al.*, 1983). Basierend auf einer Quantifizierung der zellulären rRNA Konzentration der *E. coli* Populationen im Mäusedarm wurde hingegen berechnet, daß die Verdopplungszeit bei etwa 1 – 2 Stunden liegen müßte (Poulsen *et al.*, 1994). Ein derart schnelles Wachstum war jedoch völlig unvereinbar sowohl mit der Größe der *E. coli* Population als auch der Exkretionsrate. In einer späteren Studie wurde dieses scheinbare Paradoxon gelöst (Licht *et al.*, 1999). Man fand heraus, daß es zwei verschiedene Subpopulationen gab, eine kleine schnellwachsende im Mucus und eine zweite, viel größere aber kaum wachsende Subpopulation im Lumen des Darmes. Letztere schien durch einen bisher unbekannten antibakteriellen Wirkstoff im Wachstum gehemmt zu sein. Beide Populationen wiesen ähnliche rRNA Konzentrationen auf.

Ein weiteres Problem der Aktivitätsbestimmung von Einzelzellen mit dieser Methode ist, daß lediglich die Menge akkumulierter rRNA gemessen wird, sich daraus aber keine Information über RNA-Syntheseraten ableiten läßt. Die Kontrolle der Makromolekülsynthese läuft bei Bakterien hauptsächlich über die Initiation, sei es DNA Replikation, Transkription oder Translation. Bei *E. coli* wurde gezeigt, daß nur einer der zwei rrn Promotoren, der Promotor P1, wachstumsratenabhängig kontrolliert wird (Gourse *et al.*, 1986). Aber selbst wenn man die Aktivität des P1 Promoters in Einzelzellen verfolgen könnte, spielen Degradationsraten von rRNA ebenfalls eine signifikante Rolle bei der wachstumsabhängigen Regulation des Ribosomengehaltes (Gausing, 1977). In schnell wachsenden Zellen wäre es prinzipiell möglich, durch Hybridisierung auf sogenante "internal transcribed spacer" Sequenzen (ITS), die Menge an neu synthetisierter

unreifer rRNA zu quantifizieren (Licht *et al.*, 1999; Oerther *et al.*, 2000). Die ITS Sequenzen befinden sich zwischen der 16S und der 23S rRNA im Primärtranskript des rrn Operons und werden vor der Bildung reifer Ribosomen von einer spezifischen Endonuklease entfernt. Da diese Intermediate schneller abgebaut werden als reife rRNA (Cangelosi und Brabant, 1997), kann man durch quantitative FISH auf ITS die rRNA Syntheseaktivität genauer bestimmen. Leider ist dieser Ansatz in marinen Habitaten derzeit nicht anwendbar, da das Primärtranskript in wesentlich geringerer Zahl als die reife rRNA vorliegt (ca. 5% [Cangelosi und Brabant, 1997]) und FISH schon gegen reife rRNA sensitivitätslimitiert ist (Eilers *et al.*, 2000b). FISH mit einfach markierten Oligonukleotiden ist somit gegenwärtig bei weitem nicht sensitiv genug, um Zellen mit einer derart niedrigen Zahl an Zielmolekülen anzufärben.

E. coli kann 70S Ribosomen zu 100S Ribosomen dimerisieren und somit inaktivieren und speichern. Es wird angenommen, daß 100S Ribosomen essentiell für das Überleben von *E. coli* in der stationären Phase sind (Wada *et al.*, 1995), da während Hungerperioden Ribosomen als Energie- und Nährstoffquelle genutzt werden können. Für verschiedene Mikroorganismen konnte gezeigt werden, daß bei Kohlenstofflimitierung der Abbau der rRNA beginnt (Kjelleberg, 1993). Wenn hingegen extrazelluläre Produkte oder Reservestoffe vorhanden sind, kann dieser RNA-Abbau verlangsamt werden, beim Entfernen dieser Produkte durch Auswaschung setzte der RNA Abbau sofort wieder ein (Strange *et al.*, 1961; Dawes und Ribbons, 1965; Burleigh und Dawes, 1967; Boylen und Ensign, 1970; Scherer und Boylen, 1977; Nazly *et al.*, 1980). Während des Abbaus von RNA erfolgte eine Akkumulation UV-absorbierenden Materials (Purine und Pyrimidine) sowie anorganischen Phosphates (Strange *et al.*, 1961; Postgate und Hunter, 1962). Die freigesetzte Ribose diente als Hauptenergiequelle während der Kohlenstofflimitierung. Während Phosphorlimitierung kann RNA auch als Hauptphosphorquelle dienen: Sind die zelleigenen Phosphorquellen verbraucht, wie z.B. Orthophosphate oder Phospholipide, wird RNA schnell abgebaut, und das neu verfügbare Phosphat kann dann für die DNA Synthese verwendet werden. So konnte bei Phosphorlimitierung ein Transfer von bis zu 60% des Phosphors aus der RNA in die DNA mit Hilfe von [^{32}P]-markierter RNA nachgewiesen werden (Horiuchi, 1959; Medveczky und Rosenberg, 1971).

Bei Untersuchungen an marinen Bakterien während der stationäre Phase wurde festgestellt, daß in manchen Stämmen die Ribosomen über eine längere Zeit gespeichert werden (Flärdh *et al.*, 1992; Kerkhof und Kemp, 1999). Andere hingegen bauen mit dem Ende der logaritmischen Wachstumsphase ihre Ribosomen schnell ab (Kerkhof und Kemp, 1999;

Oda *et al.*, 2000). Es wird vermutet, daß einige "opportunistische" Bakterien eine hohe Ribosomenkonzentration während Nicht-Wachstums beibehalten, um durch eine potentiell hohe Kapazität zur Proteinbiosynthese schneller auf Änderungen der Wachstumsbedingungen reagieren zu können (Flärdh *et al.*, 1992; Fegatella *et al.*, 1998).

2. *In situ* Quantifizierung mariner Mikroorganismen

Die Fluoreszenz *in situ* Hybridsierung (FISH) von Bakterien wurde vor mehr als 10 Jahren zum ersten Mal beschrieben (DeLong *et al.*, 1989; Amann *et al.*, 1990) und als ein Durchbruch für die mikrobielle Ökologie gefeiert. Leider mußte man bald feststellen, daß in Umweltproben, die nicht aus hocheutrophen Systemen stammen, die Identifizierung von Bakterien durch FISH oft problematisch ist. Die meisten heterotrophen Bakterien aus dem marinen Pelagial sind klein, langsam wachsend oder dormant (Morita, 1997). Saisonal können daher die Signalintensitäten eines Großteils der hybridisierten Bakterien im Plankton der Deutschen Bucht unterhalb der Detektionsgrenze liegen (Eilers *et al.*, 2000b; Eilers *et al.*, 2001).

In den letzten Jahren wurden verschiedene Möglichkeiten vorgestellt, um die Sensitivität der FISH zu verbessern: hellere Fluorochrome (Alfreider *et al.*, 1996; Glöckner *et al.*, 1996), Restlichtverstärkung und Bildanalyse (Fuhrman und Ouverney, 1998), Inkubation mit Chloramphenicol zur Erhöhung des rRNA Gehaltes der wachsenden Bakterienfraktion (Ouverney und Fuhrman, 1997), gleichzeitige Hybridisierung mit mehreren fluoreszenzmarkierten Oligonukleotidsonden (Lee *et al.*, 1993), unmarkierte Helferoligonukleotide (Fuchs *et al.*, 2000; Glöckner *et al.*, 2000), mehrfach markierte Polyribonukleotidsonden (siehe 2.1.) (DeLong *et al.*, 1999; Karner *et al.*, 2001) und enzymatische Signalverstärkung (siehe 2.2.) (Lebaron *et al.*, 1997; Schönhuber *et al.*, 1997).

2.1. Hybridisierung mit mehrfach fluoreszenzmarkierten Polyribonukleotidsonden

Gen-spezifische Ribonukleotidsonden können durch in vitro Transkription von PCR-generierten DNA-Vorlagen synthetisiert werden, welche einen RNA-Polymerase-Promotor (T3, T7) am 5' Ende aufweisen. Während der Transkription werden markierte Nukleotide zugesetzt. Dadurch kann eine mit zahlreichen Reportermolekülen versehene Sonde synthetisiert werden.

In der Mikrobiologie wurden fluoreszenzmarkierte Polyribonukleotidsonden vor 8 Jahren zum ersten Mal für FISH von Bakterienzellen in Rein- und Mischkultur beschrieben (Ludwig *et al.*, 1994; Trebesius *et al.*, 1994). Eine bis zu 26-fache Steigerung der Signalintensität hybridisierter Zellen konnte mit dieser Methode erreicht werden, verglichen mit einfach markierten Oligonukleotidsonden. Erst in jüngster Zeit wurde dieser Ansatz für die Detektion von Mikroorganismen im marinen Picoplankton angepaßt (DeLong *et al.*, 1999). Es wurden Polyribonukleotidsonden entwickelt, mit deren Hilfe man zwischen *Bacteria*, *Crenarchaeota* und *Euryarchaeota* unterscheiden konnte. Auch in diesem Fall gab es Hinweise auf eine signifikante Erhöhung der Signalintensitäten sowie der relativen Abundanz hybridisierter Zellen, die allerdings nicht systematisch belegt wurden. Die eingesetzten Sonden waren Transkripte von ganzen 16S und 23S rDNA Genen, welche entweder aus extrahierter DNA aus dem Pazifik stammten (*Bacteria*), oder von großen klonierten DNA-Fragmenten (Fosmiden), die 16S und 23S rDNA enthielten (*Crenarchaeota*, *Euryarchaeota*). Diese sehr langen (> 2 kb) Transkripte wurden anschließend in kleinere Fragmente (0.1 kb) hydrolysiert. Mit Hilfe dieser Methode konnte gezeigt werden, daß selbst in tiefen Wasserschichten die meisten Zellen, die mit einem allgemeinen DNA-Farbstoff markierbar waren (4',6-diamidino-2-phenylindol, DAPI), auch mit der FISH detektierbar sind. Somit enthält der Großteil aller Bakterioplanktonzellen auch signifikante Mengen an rRNA, und kann daher nicht als beschädigte oder leere Zellhüllen („ghosts") (Zweifel und Hagström, 1995; Heissenberger *et al.*, 1996) betrachtet werden.

2.2. Enzymatische Signalverstärkung

Eine Signalverstärkung in mikroskopischen Präparaten durch katalysierte Anlagerung von Reportermolekülen ("catalyzed reporter deposition", CARD) wurde erstmals 1989 von Bobrow et al. für Immunoblots und ELISAs (Enzyme Linked Immuno Sorbent Assays) vorgestellt (Bobrow *et al.*, 1989). CARD basiert auf der Anlagerung einer großen Zahl markierter Tyramidmoleküle durch die Aktivität der Meerrettichperoxidase (horseradish peroxidase, HRP). Für CARD-Signalverstärkung ist die HRP mit einem Detektormolekül (Antikörper, Oligonukleotid) konjugiert. Dadurch wird sie in einem ersten Färbeschritte spezifisch in der Zielregion gebunden, z.B. an bakterielle Ribosomen, und entfaltet dort ihre katalytische Wirkung. Tyramide sind phenolische Verbindungen, welche, wenn sie in hohen Konzentrationen vorliegen, durch die HRP dimerisiert werden können. Dies geschieht vermutlich über die Bildung freier Radikale (Zaitsu und Ohkura, 1980). Stehen

bei niedrigeren Tyramidkonzentrationen nicht genügend Reaktionspartner zur Verfügung, nimmt die Wahrscheinlichkeit einer Reaktion mit anderen Molekülen der festen Phase zu. Es erfolgt eine verstärkte Bindung der hochreaktiven Intermediate an elektronenreiche Bereiche von benachbarten Proteinen im Präparat, z.B. an Tyrosin (Abb. 1). Auf diese Weise kann eine große Menge markierter Tyramide in unmittelbarer Nähe der Peroxidasebindungsstelle kovalent gebunden werden. Die CARD-Signalverstärkung erlaubt eine Senitivitätssteigerung um bis zu 1000-fach, verglichen mit konventionellen Avidin-Biotin-Komplex-Verfahren (Adams, 1992; Berghorn *et al.*, 1994; Merz *et al.*, 1995; Sanno *et al.*, 1996).

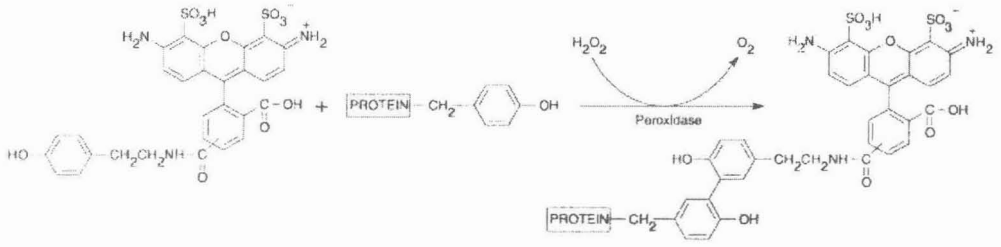


Abbildung 1. Bindung markierten Tyramids an Proteine durch die Aktivität der Peroxidase (Quelle: Molecular Probes, http://www.probes.com)

Die CARD wurde daher auch zur Visualisierung von Antigenen oder von inkorporiertem Bromdeoxyuridin in der Fluoreszenzmikroskopie (Chao *et al.*, 1996; Van Gijlswijk *et al.*, 1997; Vanheusden *et al.*, 1997) oder in der Elektronenmikroskopie (Mayer und Bendayan, 1999) verwendet. Seit 1995 wird die CARD für die Detektion von RNA und DNA Sequenzen in Gewebeschnitten und Zellpräparaten eingesetzt, in Kombination mit der *in situ* Hybridisierung . Dank der enzymatischen Signalverstärkung kann die Sensitivität der *in situ* Hybridisierung um das Doppelte bis Hundertfache gesteigert werden. Obendrein ist die CARD-Signalverstärkung auch in mehreren "Schichten" möglich: die von der HRP umgesetzten Tyramide können mit einer primären Markierung konjugiert sein (z.B. Fluorescein, Digoxygenin), die in einem darauffolgenden Färbeschritt ihrerseits das Zielmolekül für einen mit HRP-markierten Antikörpers darstellt. In einem zweiten Amplifikationsschritt können dann weitere, sekundär markierten Tyramide spezifisch angelagert werden (Speel *et al.*, 1995; Speel *et al.*, 1997a) (Abb. 2). Damit ermöglicht die Signalverstärkung u.a. die Detektion von (a) repetitiven und Einzelkopie DNA Sequenzen

(von etwa 1 bis 5 kB), (b) simultan bis zu drei verschiedene DNA Sequenzen (Speel *et al.*, 1997b; Van Gijlswijk *et al.*, 1997; Hopman *et al.*, 1998) sowie (c) Einzelkopien menschlicher Papillomaviren und anderen Mikroorganismen in Zell- und Gewebepreparaten und (d) hoch bis niedrig abundanter rRNAs und mRNAs in Zell- und Gewebepräparaten (Tabelle 1).

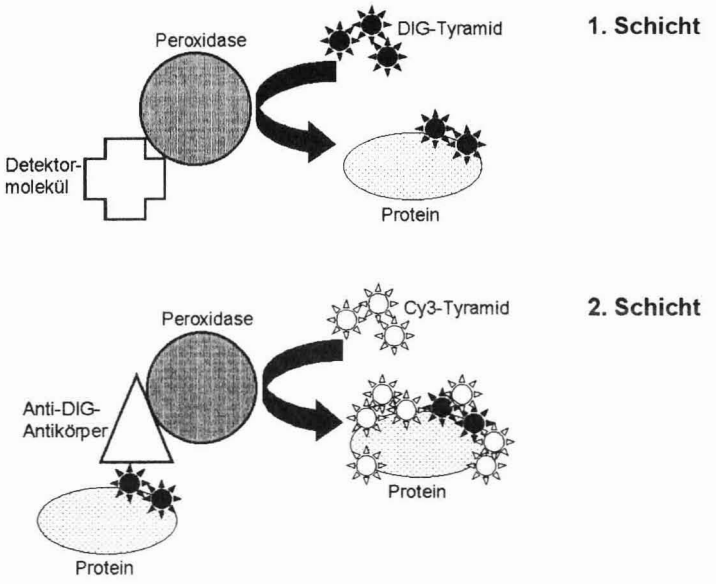


Abbildung 2. CARD in mehreren zytochemischen Detektionsschichten am Beispiel von Digoxygenin (DIG) markiertem Tyramid (1. Schicht), einer Anti-DIG-Antikörperreaktion und nachfolgender Umsetzung eines fluoreszenzmarkierten (z.B. Cy3) Tyramids (2. Schicht).

1.2.1. Fluoreszenz-*in situ*-Hybridisierung mit enzymmarkierten Oligonukleotidsonden

Die Anwendung von HRP-markierter Sonden zur spezifischen Detektion von Bakterienzellen mit niedrigem rRNA-Gehalt wurde in der Vergangenheit mehrmals als Alternative zu FISH mit einfach fluoreszenzmarkierten Oligonukleotidsonden vorgeschlagen (Amann *et al.*, 1992; Zarda, 1994; Amann *et al.*, 1997b; Lebaron *et al.*, 1997; Schönhuber *et al.*, 1997; Schönhuber *et al.*, 1999). Eine Anwendung dieser Methode für heterotrophe Mikroorganismen in Planktonproben scheiterte bisher allerdings stets an der notwendigen Permeabilisierung der Zellwand. Das Eindringen großer Moleküle wie der HRP (bzw. anderer Enzyme oder Antikörper) in formaldehydfixierte Bakterienzellen bedarf eines zumindest teilweisen Verdaus der Zellwand. Protokolle für die Permeabilisierung von Bakterienzellen beschränkten sich deshalb entweder auf qualitative Analysen von nah verwandten Mikroorganismen mit gleicher Zellwandbeschaffenheit, z.B. bei Cyanobakterien (Schönhuber *et al.*, 1999), oder aber auf bestimmte Einzelorganismen, wie *Escherichia coli* (Juretschko *et al.*, 1999), *Listeria monocytogenes* (Wagner *et al.*, 1998) und *Streptococcus suis* (Boye *et al.*, 2000) (Tabelle 1). Hingegen gab es bislang keine geeignete Fixierungs- und Vorbehandlungsmethoden für einen breiteren Einsatz der Kombination von FISH und CARD für die Identifizierung von Mikroorganismen in komplexen Umweltproben. Was fehlte war eine breit einsetzbare Technik, die zum einen auch schwer zugängliche Zellen für HRP-markierte Sonden permeabilisiert, zum anderen aber noch nicht zur Lyse der leichter zugänglichen Zellen führt. In Folge lagen die Detektionsraten von Bakterioplanktonzellen mit HRP-markierten Oligonukleotiden nicht oder nur unwesentlich höher als mit direkt markierten Sonden (Lebaron *et al.*, 1997).

Tabelle 1. Beispiele für die Kombination von CARD Signalamplifikation und *In situ* Hybridisierung

Biologisches Material[a]	Ziel DNA/RNA	Schichten der Zytochem. Detektion[b]	Tyramid Konjugat[c]	Visualisierung[d]	Referenz
Chrom.	Genomische DNA (Einzelkopie, 15kb)	3 Schichten	FITC	FM	Macechko *et al.*, 1997
Chrom. / Zellen	Genomische DNA (Einzelkopie, 5.2 und 10.7 kb)	1 Schicht	Cy3.29	FM	Schmidt *et al.*, 1997
Chrom. / Zellen	Genomische DNA (repetetiv, Einzelkopie, 15.8 und 40 kb)	1 bis 3 Schichten	Cou, FITC, Rho	FM	Speel *et al.*, 1997
Zellen	Menschliche Papilloma Virus Typ 16 DNA (293-1978 bp)	1 Schicht	Bio	StrAvTxRed, FM	Adler *et al.*, 1997
			Cy3	FM	
Zellen	Genomische DNA (repetetiv)	1 Schicht	FITC	FM	Plenat *et al.*, 1997
Zellen / Gewebe	Menschliche Papilloma Virus Typ 16 und 18 DNA	3 Schichten	Bio	ABC-DAB, BM	Poddighe *et al.*, 1996
Zellen / Gewebe	*Yersinia enterocolitica* DNA (70 kb)	3 Schichten	Bio	ABC-DAB, BM	Odinot *at al.*, 1998
Zellen	*Plasmodium falciparum* A Typ rRNA	1 Schicht	Bio	StrAvFITC, FM	Raap *et al.*, 1995
	Menschlicher Cytomegalovirus, frühe mRNA				
	Menschliche Elogationsfaktor mRNA		Cou, FITC, Rho	FM	
Zellen / Gewebe	Menschliche Herpesvirus 8 Cyclin D Homolog RNA	1 Schicht	Bio	StrAvHRP-DAB, BM	Reed *et al.*, 1998
Gewebe	Menschliche Insulin und Vasointestinalpolypeptid mRNA	1 Schicht	Bio, Dig, DNP, TNP	AbHRP-DAB, BM	Speel *et al.*, 2000
			FITC	FM	
Bakterienzellen	Bakterielle Reinkulturen & marines Bakterioplankton	1 Schicht	TRITC	OligoBIO, AbHRP	Lebaron *et al.*, 1997
Bakterienzellen	Bakterienzellen, Reinkulturen & Belebtschlamm	1 Schicht	FITC	OligoHRP, FM	Schönhuber *et al.*, 1997
Bakterienzellen	*Listeria monocytogenes* mRNA	1 Schicht	FITC	DigHRP-Fab, FM	Wagner *et al.*, 1998
Bakterienzellen	Cyanobakterien, rRNA	1 Schicht	FITC, DAB	OligoHRP, FM, BM	Schönhuber *et al.*, 1999

[a] Chrom, Chromosomen
[b] HRP, Meerettichperoxidase
[c] Bio, Biotin; Cou, Coumarin; Dig, Digoxigenin; DNP, Dinitrophenyl; FITC , Fluoresceinisothiocyanat, Rho, Rhodamin; TNP, Trinitrophenyl;
[d] ABC, Avidin-biotinylierter Peroxidasekomplex; AbHRP, HRP konjugierter Antikörper; FM, Fluoreszenzmikroskopie; BM, Hellfeldmikroskopie; DAB, Diaminobenzidin; StrAvFITC, FITC konjugiertes Streptavidin; StrAvTxRed, Texas red konjugiertes Streptavidin; DigHRP-Fab, Anti-Digoxigenin-Peroxidase Fab Fragment; OligoHRP, Peroxidase-markierte Oligonukleotidsonde; OligoBio, Biotin-markierte Oligonukleotidsonde

2.2.2. Identifizierung DNA-synthetisierender Bakterienpopulationen

Die Verwendung von radioaktiv markierten "tracer"-Molekülen wie Thymidin (TdR) oder Leucin ermöglicht eine Bestimmung der Gesamt-DNA- und -Biomasse-syntheseraten von Bakterioplanktongemeinschaften (Fuhrman und Azam, 1982; Kirchman *et al.*, 1982; Kirchman, 1993; Simon und Wunsch, 1998). Darüberhinaus kann mittels Mikroautoradiographie auch die Aufnahme dieser Substanzen in einzelne Bakterienzellen sichtbar gemacht werden (Brock, 1967; Simek, 1986; Karner und Fuhrman, 1997). Die Mikroautoradiographie ist gegenwärtig anderen sogenannten "Einzelzelltechniken" zur Bestimmung mikrobieller Aktivität, z.B. der Reduktion von Tetrazoliumsalzen, Lebend-Tot-Färbungen oder der Einteilung in Zellen mit hohen und niedrigem DNA-Gehalt (Choi *et al.*, 1996; Gasol *et al.*, 1999; Sherr *et al.*, 1999), vor allem deshalb überlegen, weil sie sich mit FISH kombinieren läßt (Lee *et al.*, 1999; Cottrell und Kirchman, 2000; Ouverney und Fuhrman, 2001) und dadurch Aussagen über die Aufnahmeaktivitäten verschiedener Bakteriengruppen ermöglicht.

Allerdings ist diese Methode auch sehr aufwendig, mit oft wochenlangen Inkubationszeiten, und hohem Arbeitseinsatz bei der mikroskopische Auswertung. Deshalb existieren bereits seit längerem nicht-radioaktive Alternativen, die sich in bestimmten Anwendungsbereichen vor allem wegen ihrer Einfachheit durchsetzten konnten. Eine typische Fragestellung, die ursprünglich mit Mikroautoradiographie untersucht wurde, ist die Quantifizierung von DNA-synthetisierenden Zellen (d.h. Zellen mit TdR-Einbau) in Zellkulturen oder Umweltproben (Brock, 1967; Simek, 1986; Pedros-Alio und Newell, 1989; Tuomi *et al.*, 1995; Andreasen und Nielsen, 1997). Bromdeoxyuridin (BrdU) ist ein halogeniertes Analogon von TdR und wird, wie TdR in neu synthetisierte DNA eingebaut. In der Histochemie und Cytochemie wird die Markierung von DNA mit BrdU ("pulse-labeling") und eine nachfolgende immunzytochemische Detektion des inkorporierten BrdUs für die Untersuchung von Proliferation in eukaryontischer Zellen intensiv genutzt (Moran *et al.*, 1985; Rizzoli *et al.*, 1988). Da der Anti-BrdU-Antikörper nur an BrdU in einzelsträngiger DNA bindet, ist mit dieser Methode eine *de novo* DNA-Synthese sogar spezifischer nachweisbar als durch TdR-Inkorporation. Sucht man in der Datenbank des *Institute of Scientific Information* nach dem Begriff "bromodeoxyuridine", werden für die letzten 10 Jahre mehr als 5200 Publikationen angegeben, etwa 400 bis 500 pro Jahr. Hingegen wurde BrdU bisher lediglich in 4 Untersuchungen an mikrobiellen Gemeinschaften eingesetzt (Borneman, 1999; Steward und Azam, 1999; Urbach *et al.*, 1999; Yin *et al.*, 2000). So wurde BrdU einmal als nicht-radioaktive Alternative zu TdR

für die Messung von Gesamtaufnahmeraten von Bakterioplankton verwendet (Steward und Azam, 1999), für die Separierung der DNA von BrdU-inkorporierenden Bakterien in DNA-Extrakten (Borneman, 1999; Urbach *et al.*, 1999; Yin *et al.*, 2000), und für immonozytochemische Färbung mariner Bakterienisolate in Reinkultur (Urbach *et al.*, 1999).

Allerdings waren die etablierten hochsensitiven Methoden für die Detektion inkoporierten BrdUs in Einzelzellen bisher nicht dazu geeignet, DNA-Synthese in Bakterienzellen aus Umweltproben nachzuweisen (Shapiro, 1995). Wie schon unter 2.1.1. erwähnt, ist auch in diesem Fall der kritische Schritt eine hinreichende Permeabilisierung der Zellhülle aller Bakterien, ohne einen Teil der Zellen durch diese Behandlung zu zerstören. Hinzu kommt, daß der Anti-BrdU-Antikörper nur an BrdU in einzelsträngiger DNA bindet (Moran *et al.*, 1985). Eine Denaturierung der DNA und die Demaskierung der Epitope durch Hitze (Shi *et al.*, 1991), DNasen (Dinjens *et al.*, 1992; Takagi *et al.*, 1993), Detergenzien (Wilson und Bianchi, 1999), und Salzsäure (Bak und Panos, 1997) kann zwar die Immunreaktivität von BrdU in formaldehydfixierten Zellen wiederherstellen, doch auch diese Schritte können gleichzeitig einen Zellverlust oder den Verlust des Zielmoleküls, der genomischen DNA, bewirken.

Eine Methode, welche die Detektion inkorporierten BrdUs in Einzelzellen mit der FISH kombiniert, wäre für die Erforschung der Ökologie planktischer Bakterien von großem Vorteil: es könnte damit nicht nur die Gesamtanzahl der in situ DNA-synthetisierende Zellen in einer Gemeinschaft bestimmt, sondern darüberhinaus auch verschiedene Populationen von BrdU-inkorporierenden Bakterien identifiziert werden. Dadurch wäre es möglich, Reaktionen verschiedener Bakteriengruppen in Umweltproben auf experimentelle oder natürliche Änderungen ihrer Lebensbedingungen zu entdecken, noch bevor sich ihre Zellzahl verändert. Für Zellkulturen und Gewebeschnitte stehen heute verschiedene Protokolle für eine simultane Färbung mit Immunzytochemie und *in situ*-Hybridisierung zur Verfügung. Mit diesen Methoden wurde z.B. die Präsenz von spezifischen mRNAs sowie ihrer Proteine in Eukaryotenzellen nachgeweisen (Larsson und Hougaard, 1991; Harper *et al.*, 1992; Heppelmann *et al.*, 1994; Dirks, 1996). Die Anforderungen, die an eine optimale Kombination von Immunzytochemie und der *in situ*-Hybridisierung gestellt werden, beinhalten die Beibehaltung der Zellmorphologie und der Epitope, die Zugänglichkeit der Nukleinsäurezielsequenzen, keine Kreuzreaktionen zwischen den verschiedenen Detektionsmethoden und einen guten Farbkontrast und Stabilität der Fluorochrome.

B

Ergebnisse und Diskussion

B

Ergebnisse und Diskussion

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

1. Messung von zellulären rRNA-Konzentrationen für die Abschätzung der Aktivität von Einzelzellen

(Annelie Pernthaler, Jakob Pernthaler, Heike Eilers, Rudolf Amann. Growth patterns of two marine isolates: Adaptations to substrate patchiness? Applied & Environmental Microbiology. **67**(9):4077-4083, 2001)

In dieser Studie wurde das Wachstumsverhalten und die Änderungen im zellulären rRNA-Gehalt zweier mariner Isolate (*Pseudoalteromonas* sp., *Oceanospirillum* sp.) in statischer Kultur untersucht. Dabei standen zwei Aspekte im Vordergrund. Erstens sollte eine Methode entwickelt werden, um Fluoreszenzintensitäten an Einzelzellen nach FISH an einem konfokalen Laserscanningmikroskop zu messen. Zweitens wurde untersucht, ob Unterschiede im Wachstumsverhalten der beiden Stämme sich mit der Entwicklung des zellulären rRNA-Gehaltes verknüpfen lassen. Spezifisch sollte eine Hypothese überprüft werden, daß das Beibehalten hoher rRNA-Konzentrationen während der stationären Phase ein Anzeichen für eine besonders "opportunistische" Wachstumsstrategie ist: Es wird vermutet, daß manche Bakterien mit den Ribosomen auch eine hohe potentielle Proteinsynthesekapazität während Hungerphasen konservieren, und dadurch über einen Wachstumsvorteil bei plötzlichem Nährstoffeintrag verfügen (Flärdh *et al.*, 1992). In Planktonproben der Deutschen Bucht unterschieden sich Bakterien aus der Verwandtschaft der beiden untersuchten Stämme nachweislich in ihrer Reaktion auf Nährstoffzugabe: Obwohl Vertreter beider Bakteriengattungen auf den angebotenen Substraten wachsen können (Eilers *et al.*, 2000b), reicherte sich *Pseudoalteromonas* sp. innerhalb von 48 h deutlich an, wohingegen die Abundanzen von *Oceanospirillum* sp. fast unverändert blieben (Eilers *et al.*, 2000a). Für die mikroskopische Quantifizierung von FISH-Fluoreszenz mußten einerseits geeignete Aufnahmebedingungen gefunden werden und andererseits die

Färbemethode soweit standardisiert werden, daß eine reproduzierbare Intensitätsmessung möglich war. Die anschließende Auswertung der digitalen Bilder erfolgte mit computerunterstützter Bildanalyse (Oerther *et al.*, 2000). Es konnte gezeigt werden, daß unter den gewählten Kultivierungsbedingungen der *Pseudoalteromonas*-Stamm auch nach Eintritt in die stationäre Phase hohe Konzentrationen an 16S rRNA beibehielt, der *Oceanospirillum*-Stamm hingegen die 16S rRNA bei Nichtwachstum schnell abbaute (Abb. 3). Außerdem konnte in der stationären *Oceanospirillum* sp. Population zwischen 2 Morphotypen mit unterschiedlicher Zellgröße und rRNA-Gehalt unterschieden werden. Messungen von DNA und Protein am Durchflußzytometer bestätigten diesen Dimorphismus, und legten außerdem nahe, daß die Zellteilung von *Oceanospirillum* sp. nach Substratzugabe v.a. in der rRNA- und proteinreichen Subpopulation stattfand.

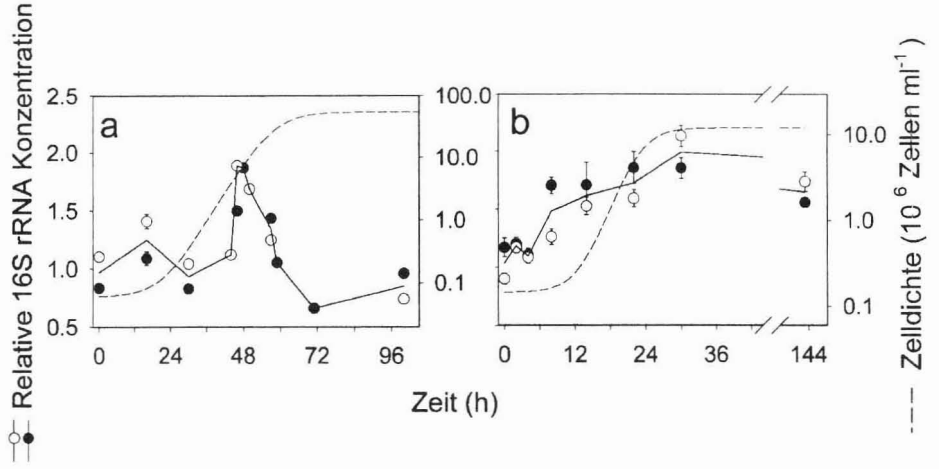


Abbildung 3. Änderung in der der zellulären 16S rRNA-Konzentrationen bei **(a)** *Oceanospirillum* sp. und **(b)** *Pseudoalteromonas* sp. in statischer Reinkultur.

Um einen spezifischen Wachstumsvorteil von *Pseudoalteromonas* sp. bei plötzlicher Nährstoffverfügbarkeit zu belegen, wurde die Zellzahländerung der beiden Stämme unter verschiedenen Substratzugabebedingungen in Kokultur untersucht. Einerseits wurde eine klassische statische Kultur mit den Stämmen beimpft, und das Wachstum beider Isolate mit der FISH mit spezifischen Sonden verfolgt. Andererseits wurden die Isolate in das substratfreie Grundmedium überführt, und die Kohlenstoffquelle über einen Zeitraum von 100 Stunden portionsweise zugegeben. Es konnte gezeigt werden, daß eine graduelle Anreicherung sich nur auf das Wachstum der *Pseudoalteromonas* sp.-

Population negativ auswirkte (Abb. 4). Ein Zusammenhang zwischen dem Beibehalten von hohen rRNA-Konzentrationen in der stationären Phase und der Fähigkeit sp. zur schnellen Reaktion auf Änderungen der Umweltbedingungen in *Pseudoalteromonas* liegt daher nahe.

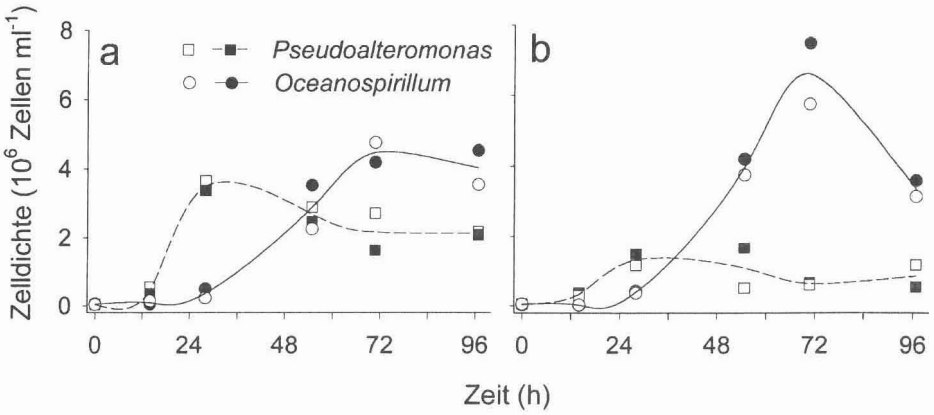


Abbildung 4. Zellzahlen von *Pseudoalteromonas* sp. und *Oceanospirillum* sp. in Kokultur **(a)** in statischer Kultur und **(b)** bei kontinuierlicher Nährstoffzugabe über 100 h

Auf dem synthetischen Meerwassermedium (MPM) nach Schut (Schut *et al.*, 1993) benötigten die marinen Isolate *Pseudoalteromonas* sp. und *Oceanospirillum* sp. 9 bzw. 18 Stunden bis zur ersten Zellteilung. Uns fiel auf, daß dieses Medium sich in den Konzentrationen einiger Salze von anderen synthetischer Meerwassermedien unterscheidet. Deshalb verglichen wir das Wachstum beider Stämme auf MPM und auf einem synthetischen Medium, welches für die Kultivierung einer marine Diatomee, *Ditylum brightwellii*, zusammengestellt wurde. Auf dem Diatomeen-Medium wuchs *Oceanospirillum sp.* deutlich schneller, d.h. es gab keinen Unterschied mehr in der Länge der lag-Phasen der beiden Isolate (Abb. 5), allerdings auch keine höheren Gesamtzellzahlen als auf MPM. Wie kann man das unverändert schnelle Wachstum von *Pseudoalteromonas* sp. auf beiden Medien erklären?

In Fischkot wurden *Pseudoalteromonas*-spezifische Bakteriophagen gefunden (A. Wichels, pers. Mitteilung), was nahelegt, daß Vertreter dieser Gattung als Darmbakterien bei Fischen auftreten. Bei Knochenfischen verändert sich die Ionenzusammensetzung oral aufgenommenen Seewassers bis zum Erreichen des Enddarms stark. Die Konzentrationen von Na^+, Cl^-, und K^+ verringern sich auf etwa 1/5, die Mg^{2+} Konzentration verdoppelt sich (Loretz, 1995). Daher ist eine bessere Anpassung von *Pseudoalteromonas* sp. an wechselnde Ionenverhältnisse vorstellbar.

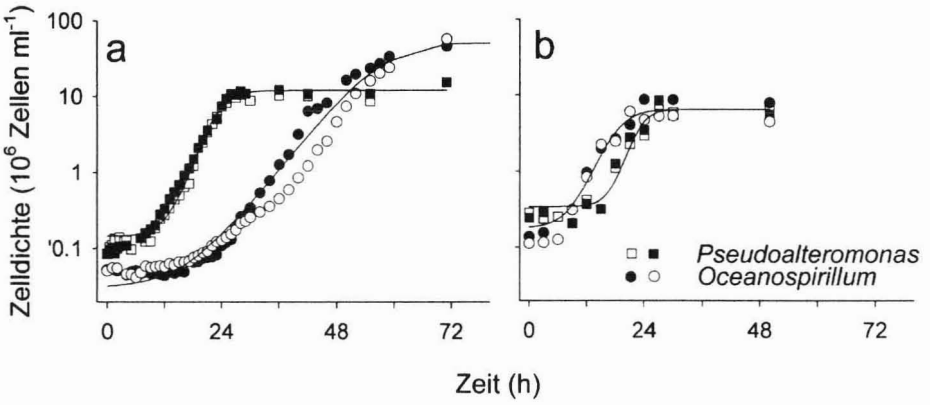


Abbildung 5. Zelldichten von *Pseudoalteromonas* und *Oceanospirillum*. Statisches Wachstum auf 2 synthetischen Merrwassermedien **(a)** MPM, **(b)** Diatomeen-Medium.

Wir untersuchten außerdem eine Abhängigkeit der Ribosomenspeicherungskapazität von *Pseudoalteromonas sp.* von der Phosphatkonzentration im Medium. Bei Wachstum ohne externe Phosphatquelle, konnte eine Abnahme der Ribosomenkonzentration bereits in der Mitte der log Phase beobachtet werden (Abb. 6). Es läßt sich vermuten, daß *Pseudoalteromonas* sp. bei Phosphatlimitierung Phosphor aus der RNA in die DNA, oder andere Zellbausteine transferiert hat (Horiuchi, 1959; Medveczky und Rosenberg, 1971).

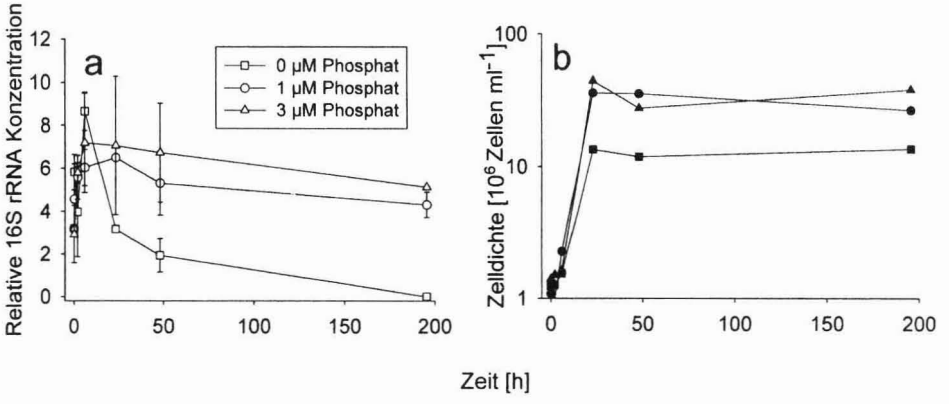


Abbildung 6. *Pseudoalteromonas* sp. während statischen Wachstums mit verschiedenen Phosphatkonzentrationen. **(a)**: rRNA Konzentrationen von Einzelzellen, **(b)**: Zellzahlen. Fehlerbalken geben die Bereiche von 2 Parallelen an.

Zusammenfassend muß gesagt werden, daß die Quantifizierung von rRNA Konzentrationen in Einzelzellen wahrscheinlich keine verallgemeinerbaren Rückschlüsse auf die Aktivität von Bakterien in Umweltproben zuläßt, sondern nur auf der Ebene von einzelnen Populationen unter kontrollierten Wachstumsbedingungen sinnvoll interpretierbar ist. Bakterien die fähig sind, Ribosomen unter bestimmten Wachstumsbedingungen zu speichern, könnten diese unter anderen Bedingungen abbauen, wie z.B. bei Phosphatlimitation (Horiuchi, 1959; Medveczky und Rosenberg, 1971) (Abb. 6). Bei Eintritt in die stationäre Phase wurde bei *E. coli* ein schneller Verlust der Ribosomen beobachtet (Davis *et al.*, 1986). Ein Teil der noch vorhandenen Ribosomen wird bei *E.coli* zu 100S Partikeln dimerisiert (Davis *et al.*, 1986). Davis et al. (1986) zeigte, daß Ribosomen in Phosphat-limitierten *E.coli* Zellen zwar vorhanden, aber defekt sind und durch die Zugabe von Initiationsfaktoren wieder Translationsaktivität zeigen. Es wird vermutet, daß der Ribosomenverlust während einer Hungerperiode der Hauptgrund für Zelltod ist (Davis *et al.*, 1986). Andererseits kann *E. coli*, das typischerweise seine Ribosomenkonzentration in der stationären Phase schnell reduziert, diese z.B. nach Kälteschock für längere Zeit konstant halten (Abb. 7), was auch mit unseren Methoden gezeigt werden konnte.

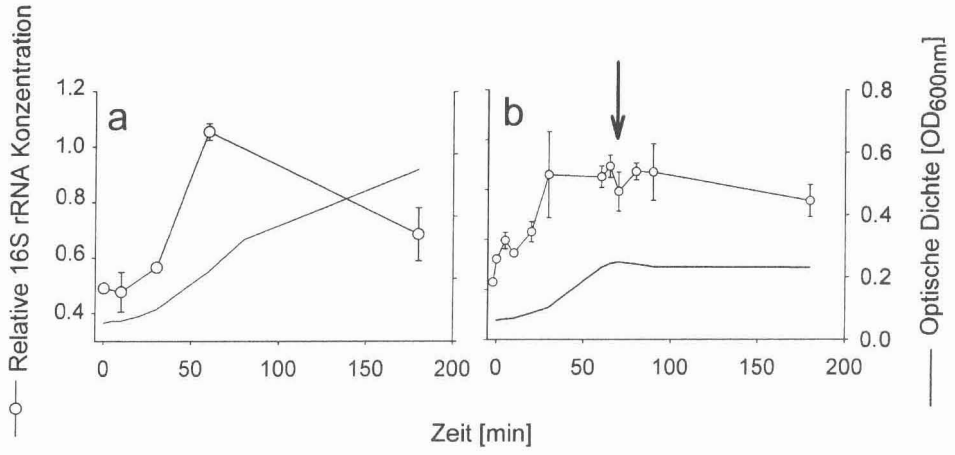


Abbildung 7. Zelluläre Ribosomenkonzentration bei *E. coli* während verschiedener Wachstumsphasen. **(a)** statisches Wachstum; **(b)** Abbruch des Wachstums und Beibehaltung hoher 16S rRNA-Konzentrationen nach Kälteschock (Pfeil).

Verglichen mit *E. coli* erfolgt der Ribosomenabbau bei *Vibrio* sp. S14 viel langsamer (Flärdh *et al.*, 1992). In Kohlenstoff-limitierten Zellen wurde gezeigt, daß mehr Ribosomen vorhanden waren als für die Translation benötigt. Zum Beispiel wurden bei etwa 1% der Gesamtproteinbiosyntheserate mehr als 50% von intakten Ribosomen nachgewiesen, verglichen mit Werten während des Wachstums. In Extrakten von S30 Partikeln (d.h. der kleinen ribosomalen Untereinheit) konnte eine Polyphenylalanin-Syntheserate gemessen werden, die vergleichbar mit Ribosomen aus wachsenden Zellen war (Flärdh, unpublizierte Resultate [Kjelleberg, 1993]). Es wird vermutet, daß dieser Vorrat an Ribosomen für einen schnellen Wachstumsstart bei Nährstoffzugabe verantwortlich ist.

Wir zeigen, daß zwei Bakterienstämme unterschiedliche Muster der zellulären 16S rRNA-Konzentration während der Wachstumsphasen aufweisen, und daß diese Muster sich bei einem *Pseudoalteromonas* sp.-Stamm unter verschiedenen Kultivierungs-bedingungen unterscheiden können. Deshalb erscheint es schwierig, in einer "Momentaufnahme" aus den zellulären rRNA-Konzentrationen allgemeine Aussagen über Wachstum oder Nichtwachstum in einer komplexen mikrobiellen Gemeinschaft zu treffen.

2. Quantifizierung mariner *Bacteria* und *Archaea* mit mehrfach fluoreszenz-markierten Polyribonukleotidsonden

(Annelie Pernthaler, Christina M. Preston, Jakob Pernthaler, Edward F. DeLong, Rudolf Amann. Comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Applied & Environmental Microbiology. 68(2):661-667, 2002)

In einer weiteren Studie wurden Detektionsraten von Bakterien- und Archaeenzellen der Nordsee und in der Monterey Bay (Kalifornien) nach FISH mit rRNA gerichteten Oligo- und Poly(ribo)nukleotidsonden, sowie deren Fluoreszenzintensitäten und ihr Bleichverhalten verglichen. In Proben aus einem Jahresgang des Bakterioplanktons im Oberflächenwasser der Deutschen Bucht bei Helgoland waren die Anteile hybridisierter Bakterien mit mehrfach markierten Polynukleotidsonden insgesamt signifikant höher als mit einfach markierten Oligonukleotidsonden. Im Winter nahmen die relativen Anteile der Zellen, die mit den Oligonukleotidsonden hybridisiert werden konnten, deutlich ab. Eine vergleichbare Abnahme wurde bei Hybridisierung mit Polynukleotidsonden nicht beobachtet (Abb. 8a). In Tiefenprofilen aus der Monterey Bay wurden die Unterschiede zwischen beiden Methoden noch deutlicher. In tieferen Wasserschichten, unabhängig von

der Jahreszeit, waren die Detektionsraten von Oligonukleotidsonden, besonders für Bakterien, sehr viel niedriger als die Detektionsraten von Polynukleotidsonden. Helligkeitsmessungen an Bakterien nach FISH mit Oligo- und Polynukleotidsonden zeigten, daß die höheren Detektionsraten der Polynukleotidsonden weniger auf größeren Signalintensitäten beruhen, sondern auf einem höheren Signal-zu-Hintergrund-Verhältnis und dem quasi-linearen Bleichverhalten des Farbstoffs Fluoresceinisothiocyanat.

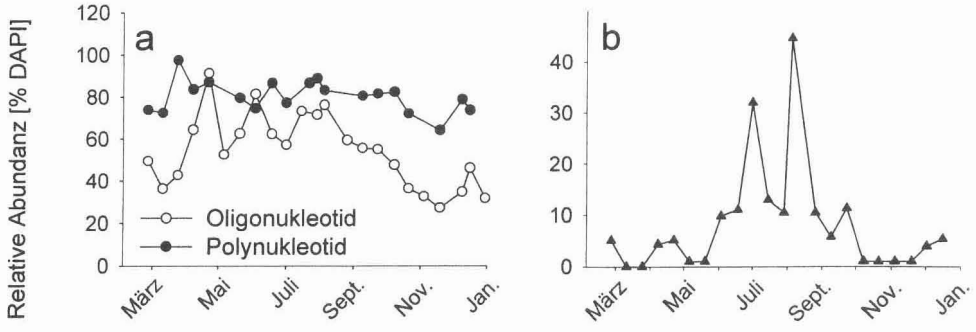


Abbildung 8. (a) Detektionsraten von Bakterien mit Oligonukleotid- und Polyribonukleotidsonden im Oberflächenwasser der Deutschen Bucht im Jahresgang. **(b)** Relativer Anteil von marinen *Euryarchaeota* am heterotrophen Picoplankton der Deutschen Bucht.

Die relativen Abundanzen von Archaeen in der Nordsee und in der Monterey Bay waren nach Hybridisierung mit Oligonukleotidsonden manchmal höher als nach Hybridisierung mit Polynukleotidsonden. Eine Doppelhybridisierung mit spezifischen Oligonukleotidsonden für *Bacteria* und *Archaea* zeigte , daß die Sonde ARCH915 für die *Archaea* auch unspezifisch einige Bakterien färbte. Allerdings konnten wir im küstennahen Oberflächenwasser der Nordsee auch mit den archaeenspezifischen Polynukleotidsonden eine Archaeenblüte während der Frühlings- und Sommermonate beobachten. Die Archaeen der sogenannten "marinen Gruppe II", welche den *Euryarchaeota* angehören (DeLong *et al.*, 1999), bildeten bis zu 30% der gesamten Picoplanktonabundanz (Abb. 8b).

Hybridisierung mit mehrfach markierten Polynukleotiden ist demnach besser geeignet für die Detektion von Bakterien mit niedrigen Ribosomengehalt in Planktonproben als FISH mit einfach fluoreszenzmarkierten Oligonukleotiden. Doch gibt es auch Nachteile dieser Technik: Die Polynukleotidsonden sind nicht kommerziell erhältlich, sondern müssen mittels PCR und in vitro Transkription selbst synthetisiert

werden. Deshalb unterscheidet sich jede Sondencharge leicht in ihrer Qualität. Außerdem sind die Polyribonukleotidsonden deutlich empfindlicher als DNA-Oligonukleotidsonden und werden als RNA-Moleküle weitaus schneller abgebaut. Obendrein können die Polynukleotidsonden, die derzeit im marinen Picoplankton eingesetzt werden, nur zwischen Mikroorganismengruppen unterscheiden, die phylogenetisch sehr weit voneinander entfernt liegen, d.h. zwischen *Bacteria*, *Crenarchaeota* und *Euryarchaeota*. Sonden von 150 – 350 Nukleotiden Länge könnten für engere phylogenetische Gruppen auf die hoch variable Regionen in der Domäne 3 der 23S rRNA entwickelt werden (Ludwig *et al.*, 1994; Trebesius *et al.*, 1994). Allerdings ist der Aufwand, um die Spezifität solcher Polynukleotidsonden zu testen, um einiges höher als bei Oligonukleotidsonden, und außerdem kann bei Erhöhung der Hybridisierstringenz (d.h. der Spezifität) die überlegene Fluoreszenzintensität der Polynukleotidsonde wieder verloren gehen (Trebesius *et al.*, 1994). Daher werden Oligonukleotide wahrscheinlich immer eine bessere phylogenetische Auflösung ermöglichen. Es schien daher insgesamt sinnvoll, eine FISH Methode zu entwickeln, welche die höhere Sensitivität der Polynukleotidsonden mit der Flexibilität und Robustheit der Oligonukleotidsonden verbinden kann (siehe Kapitel 3).

3. Fluoreszenz-*in situ*-Hybridisierung mit enzymmarkierten Oligonukleotidsonden und Tyramid-Signalverstärkung

(Annelie Pernthaler, Jakob Pernthaler, Rudolf Amann. Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria. Applied & Environmental Microbiology. In press.)

Wie in der Einleitung ausgeführt, war die breite Einsetzbarkeit der FISH mit enzymmarkierten Sonden und anschließender Tyramid-Signalverstärkung bisher in Umweltproben nicht möglich (Schönhuber *et al.*, 1997). Der kritische Schritt, die Diffusion großer Moleküle (Enzyme, Antikörper) in ganze fixierte Zellen macht es nötig, die Zellhülle vorher zu permeabilisieren. Da aber die Zellwanddicke und -beschaffenheit innerhalb der Prokaryonten und in unterschiedlichen Wachstumszuständen sehr variabel sein kann, gab es bislang keine Permeabilisierungsstrategie, die einerseits auch schwer zugängliche Zellen permeabilisiert, zum anderen aber noch nicht zur Lyse der leichter zugänglichen Zellen führt.

In Rahmen dieser Studie entwickelten wir ein neues Protokoll für die Permeabilisierung und die nachfolgende FISH von planktischen und benthischen marinen

Bakterien mit Meerrettichperoxidase-markierten Oligonukleotidsonden. Dank eines neuen Einbettverfahrens in niedrigschmelzende Agarose konnten wir Zellen auf Filtern konzentrieren und anschließend mit hohen Konzentrationen von Lysozym behandeln, ohne daß ein merklicher Zellverlust nachweisbar war (Abb. 9).

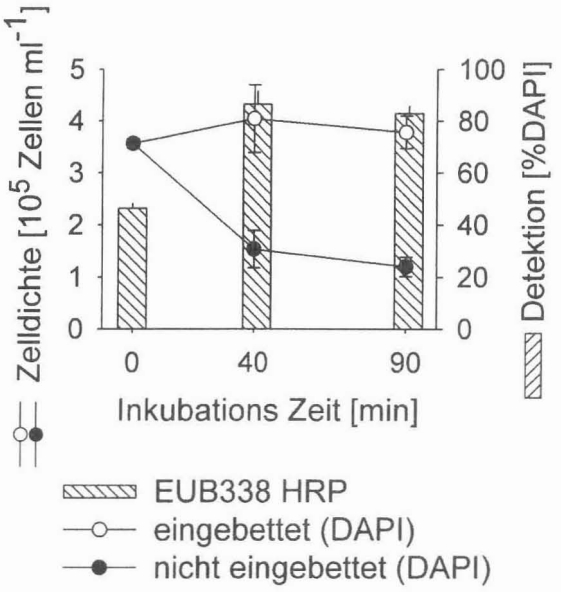


Abbildung 9. Einfluß des Lysozymverdaus auf die Zelldichten von Bakterioplanktonzellen und die FISH Detektionsraten. Die Zellen befanden sich auf Membranfiltern, die entweder in Agarose eingebettet wurden oder uneingebettet waren. Balken: %FISH Detektionsraten mit einer allgemeinen HRP-markierten Oligonukleotidsonde (EUB338) und CARD in eingebetteten Proben. Linien: Zelldichten (DAPI). Fehlerbalken zeigen entweder die Standardabweichungen (Zelldichten) oder die Bereiche (% Detektion) von Triplikaten an.

Nach dieser Vorbehandlung waren die Bakterienzellen so permeabilisiert, daß eine Hybridisierung mit enzymmarkierten Sonden möglich wurde. Verglichen mit einfach fluoreszenzmarkierten Oligonukleotidsonden, konnten wir in Plankton- und Benthosproben aus der Deutschen Bucht eine signifikante Erhöhung der Detektionsraten und der Signalintensitäten hybridisierter Zellen nachweisen (Abb. 10). Mit der neuen FISH-Methode wurde außerdem im Plankton der Deutschen Bucht eine Population der marinen SAR86 Gruppe nachgewiesen, die im Mittel >7% der Gesamtzellzahl bildete. Diese sehr

kleinen SAR86-Zellen mit höchstwahrscheinlich geringem zellulären rRNA-Gehalt konnten mit einfach fluoreszenzmarkierten Sonden nicht detektiert werden (Tab. 2).

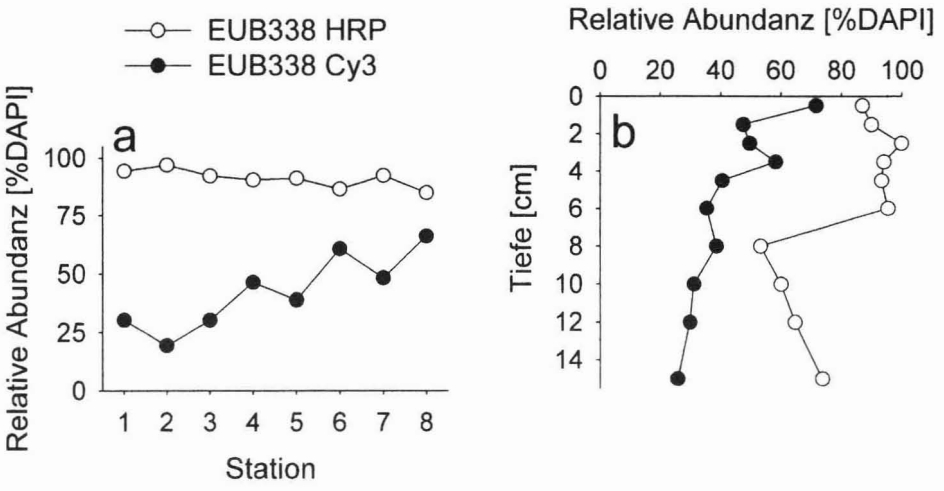


Abbildung 10. Vergleich der Detektionsraten von Bakterien nach FISH mit einfach markierten Oligonukleotidsonden oder enzymmarkierten Sonden und Tyramid Signalverstärkung. **(a)** Planktonproben, Transekt Helgoland-Cuxhaven, Oktober 1999, **(b)** Wattenmeersediment, Dangast, Februar 2000

Tabelle 2. Relative Abundanz (% DAPI) von Bakterien, hybridisiert mit einer HRP-markierten Oligonukleotidsonde spezifisch für SAR86. Mittelwerte von 8 bzw. 6 Stationen eines Transektes (Informationen zu den Transekten: siehe Teil II, Kapitel 3)

	Mittelwert	Min. / Max.
Eider – Helgoland	7.6	5.1 / 10.9
Helgoland – Cuxhaven	5.9	2.9 / 7.0
Helgoland – Nordsee	8.4	4.9 / 7.3

Das Potential der Enzymmarkierung und anschließenden Tyramid-Signalverstärkung ist allerdings nicht nur auf die Hybridisierung von rRNAs begrenzt. Wenn ganze fixierte Zellen prinzipiell für Enzyme und/oder Antikörper durchlässig gemacht werden können, eröffnen sich viele Möglichkeiten. Andere Färbemethoden, die in der Histologie und Zytochemie bereits Routine sind, könnten damit auch für

Umweltmikrobiologen verfügbar werden. Damit rückt z.B. die Detektion von mRNAs in ganzen Bakterienzellen aus Umweltproben in den Bereich des Möglichen, oder der intrazelluläre Einsatz von Antikörpern zur zytochemischen Detektion von DNA-Synthese (siehe Kapitel 4).

4. Identifizierung DNA-synthetisierender Populationen im Bakterioplankton der Nordsee

(Annelie Pernthaler, Jakob Pernthaler, Martha Schattenhofer, Rudolf Amann. Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton. Submitted.)

Im Rahmen dieser Arbeit wurde ein Protokoll für die immunzytochemische Färbung von inkorporiertem Bromdeoxyuridin (BrdU) in die DNA von Bakterioplanktonzellen, und deren Identifizierung mittels FISH entwickelt und getestet. Um die Sensitivität beider Methoden zu erhöhen, wurden sowohl FISH als auch die Detektion von BrdU mit Meerettichperoxidase und Tyramid-Signalverstärkung verbunden (Speel *et al.*, 1997a). Die FISH mit spezifischen enzymmarkierten Oligonukleotidsonden erfolgte vor der Antikörperreaktion. Da während der Signalverstärkung die fluoreszenzmarkierten Tyramide kovalent an Proteine gebunden werden, war es möglich, das spezifische FISH-Signal trotz mehrfacher anschließender Waschschritte zu erhalten. Um eine zweite, unabhängige enzymatische Signalamplifikation zu ermöglichen mußte allerdings die Peroxidase aus der FISH-Färbung vor dem nächsten Färbeschritt mit 0.01 M HCl zerstört werden. Ausreichende Permeabilisierung nach Agaroseeinbettung ermöglichte das Passieren eines direkt mit der Peroxidase konjugierten Anti-BrdU-Antikörperfragments (Größe 90 kDa) in die fixierte Zelle. Durch gezielte Epitopfreilegung konnte dieser Antikörper an das in die DNA inkorporierte BrdU binden.

Das Protokoll wurde an 22 Bakterienisolaten aus der Deutsche Bucht aus verschiedenen phylogenetischen Gruppen, sowie an *E.coli* getestet (Tab. 3). In allen Stämmen konnte BrdU-Inkorporation in die DNA nachgewiesen werden. Negativkontrollen aller Isolate (ohne BrdU Zugabe) sowie eine mit Nalidixinsäure (DNA-Gyrase-Hemmer) behandelte *E. coli* Kultur zeigten keine falschpositiven Signale mit dem Anti-BrdU-Antikörperfragment. Anschließend wurden Planktonproben aus der Deutschen Bucht (1.2 µm vorfiltriert, Beprobung am 24. September 2001) 20 Stunden lang bei 10°C mit bzw. ohne BrdU inkubiert. Bereits nach 4 Stunden konnte in 3-4% aller Zellen BrdU-Inkorporation und damit DNA-Synthese nachgewiesen werden. Der Anteil an BrdU-

positiven Zellen erhöhte sich während der Inkubationszeit auf über 14%, ohne nachweisbare Änderung in der Gesamtzellzahl (Abb. 11a). In Proben ohne BrdU-Zugabe lag die Häufigkeit von falschpositiven Signalen unterhalb der Nachweisgrenze (< 1 Zelle in 10 mikroskopischen Gesichtsfeldern).

Tabelle 3. Bakterielle Isolate, die auf die Fähigkeit BrdU zu inkorporieren, getestet wurden.

Isolat Nummer	Accession Nummer	Verwandschaft (16S rDNA)	Gruppe[1)]	%Detektion[2)]
0234A	AF235124	*Flavobacterium columnare*	CFB	> 90%
02ds22	AF235114	*Cytophaga uliginosa*		100%
11ds02	AF235111	*Cytophaga marinoflava*		100%
0803	AF235117	*Flavobacterium salegense*		100%
11ds10	AF239707	NOR6 Gruppe	γ-Proteobakterien	100%
KT71	AY007676	NOR5 Gruppe		> 90%
0246	AF173966	*Shewanella* sp.		100%
0919	AF173964	*Colwellia psychrophila*		> 90%
0903	AF235119	*Pseudoalteromonas atlantica*		100%
0910	AF173963	*Pseudoalteromonas halopla.*		100%
KT15	-	*Polaribacter* sp.		80-90%
0924	-	*Oceanospirillum* sp.		> 90%
0232	AF235125	*Alteromonas* sp.		> 90%
1111	AF173968	*Halomonas* sp.		> 90%
0901	AF172840	*Vibrio splendidius*		> 90%
0248	AF235127	*Photobacterium*		100%
11ds07	AF235112	NOR1 Gruppe		100%
1114	AF235108	NOR2 Gruppe		100%
1115	AF2315113	*Micrococcus* sp.	*Actino-bacteria*	30-40%
1110	AF239706	NOR7 Gruppe		100%
ATCC11775[T]	-	*Escherichia coli*		100%

[1)] CFB, *Cytophaga / Flavobacterium / Bacteroides* - Phylum

[2)] Signale nach der Anti-BrdU-HRP Antikörperreaktion, prozentualer Anteil an der Gesamtzellzahl.

Mit spezifischen FISH-Sonden wurde in drei Bakteriengruppen der Einbau von BrdU in die DNA *in situ* nachgewiesen: *Roseobacter* sp., *Alteromonas* sp. und SAR86. Während 20 h Inkubation zeigte ein steigender Prozentsatz der *Roseobacter* sp. Zellen auch BrdU Einbau, obwohl sich die Zellzahlen in dieser Gruppe nicht veränderten. Einige *Roseobacter* sp. des Planktons der Deutschen Bucht konnten bisher aus Filtraten und Verdünnungskulturen ohne zusätzliche Nährstoffzugabe angereichert und isoliert werden (Eilers *et al.*, 2001). Es ist daher naheliegend, daß in den Inkubationen eine erste Aktivierung von Zellen aus dieser Gruppe beobachtet wurde. In *Alteromonas* sp. erhöhte

sich eingangs, d.h. zwischen 4 und 8 h Inkubation, der Anteil BrdU-positiver Zellen, und in den darauffolgenden 12 h war eine deutliche Zellzahlzunahme in dieser Population zu beobachten. Dieser Befund deckt sich auch mit früheren Beobachtungen über die schnelle Anreicherung von *Alteromonas* sp. in Nordseewasser-Filtraten (Eilers *et al.*, 2000b). Nach 4 Stunden Inkubation konnte in 23% der Zellen aus der SAR86-Gruppe BrdU-Inkorporation gezeigt werden (Abb. 11b). Das ist ein deutlicher Hinweis darauf, daß die Population zum untersuchten Zeitpunkt auch *in situ* DNA-Synthese betrieben hat. Obwohl SAR86 nur 8% zur Gesamtbakterienabundanz beitrug, bildete diese Population fast die Hälfte aller Zellen mit meßbarer BrdU-Inkorporation. Im Genom von SAR86 findet sich ein Gen für ein bakterielles Proteorhodopsin und es gibt Belege, daß Vertreter der SAR86-Gruppe Phototrophie zur Energiegewinnung nützen können (Beja *et al.*, 2000; Beja *et al.*, 2001). Im Untersuchungszeitraum ist die Phytoplanktondichte, und damit die Primärproduktion, in der Deutschen Bucht typischerweise sehr niedrig (Eilers *et al.*, 2001). Es erscheint daher plausibel, daß eine Bakteriengruppe mit der Fähigkeit zur autotrophen Energiegewinnung zu dieser Jahreszeit einen großen Anteil aller aktiven Bakterien darstellt. Für einen Zusammenhang zwischen Licht und Wachstum bei SAR86 spricht darüberhinaus, daß die Zellzahlen dieser Population bereits nach 20 Stunden Dunkelinkubation deutlich abnahmen.

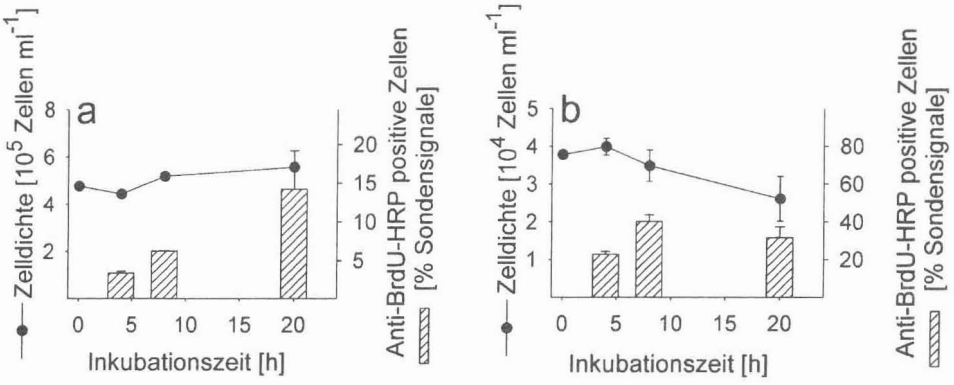


Abbildung 11. Detektion von inkorporiertem BrdU in Bakterioplanktonzellen der Nordsee. **(a)** BrdU inkorporierende *Bacteria* in [% EUB338] **(b)** BrdU inkorporierende SAR86 [in % SAR86-1249].

Diese Untersuchung liefert erste Hinweise, daß die immunzytochemische Färbung von eingebautem BrdU Aussagen über DNA-Synthese in verschiedenen bakteriellen Populationen des Nordseeplanktons ermöglicht. Alle getesteten Stämme waren in der Lage, BrdU einzubauen und in allen Stämmen waren die Permeabilisierung und Epitopfreisetzung ausreichend für die anschließende immunzytochemische Detektion von BrdU. In weiterführenden Studien soll geklärt werden, wie hoch der Anteil von BrdU-inkorporierenden Zellen im Nordseeplankton zu anderen Jahreszeiten ist und in welchen bakteriellen Populationen während und nach Phytoplanktonblüten DNA-Synthese gezeigt werden kann. Außerdem soll an Bakteriengruppen, die derzeit noch nicht kultiviert werden können (wie z.B SAR86), genauer untersucht werden, welche Art von Inkubationsbedingungen zu einer Erhöhung der DNA-Syntheseaktivität in diesen Gruppen führt.

C

Literaturverzeichnis

Literaturverzeichnis

Adams, J. C. (1992). Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. Journal of Histochemistry & Cytochemistry **40**: 1457-1463.

Adler, K., T. Erickson, M. Bobrow (1997). High sensitivity detection of HPV-16 in SiHa and CaSki cells utilizing FISH enhanced by TSA. Histochemistry Cell Biology **108**: 321-324

Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F. O. Glockner, A. Wille and R. Psenner (1996). Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Applied & Environmental Microbiology **62**(6): 2138-2144.

Amann, R., F. O. Glöckner and A. Neef (1997a). Modern methods in subsurface microbiology - *in situ* identification of microorganisms with nucleic acid probes. FEMS Microbiology Reviews **20**(3-4): 191-200.

Amann, R. and M. Kühl (1998). *In situ* methods for assessment of microorganisms and their activities. Current Opinion in Microbiology **1**: 352- 358.

Amann, R., N. Springer, W. Schönhuber, W. Ludwig, E. N. Schmid, K. D. Müller and R. Michel (1997b). Obligate intracellular bacterial parasites of acanthamoebae related to Chlamydia spp. Applied & Environmental Microbiology **63**(1): 115-121.

Amann, R. I., L. Krumholz and D. A. Stahl (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology **172**(2): 762-770.

Amann, R. I., W. Ludwig and K. H. Schleifer (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews **59**(1): 143-169.

Amann, R. I., B. Zarda, D. A. Stahl and K. H. Schleifer (1992). Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Applied & Environmental Microbiology **58**(9): 3007-11.

Andreasen, K. and P. H. Nielsen (1997). Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. Applied & Environmental Microbiology **63**(9): 3662-3668.

Azam, F. (1998). Microbial control of oceanic carbon flux: the plot thickens. Science **280**: 694-696.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil and F. Thingstad (1983). The ecological role of water-column microbes in the sea. Marine Ecology Progress Series **10**: 257-263.

Bak, P. M. and R. J. Panos (1997). Protease antigen recovery decreases the specificity of bromodeoxyuridine detection in formalin-fixed tissue. Journal of Histochemistry & Cytochemistry **45**(8): 1165-1170.

Beja, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich and E. F. DeLong (2000). Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. Science **289**(5486): 1902-1906.

Beja, O., E. N. Spudich, J. L. Spudich, M. Leclerc and E. F. DeLong (2001). Proteorhodopsin phototrophy in the ocean. Nature **411**(6839): 786-789.

Berghorn, K. A., J. H. Bonnett and G. E. Hoffman (1994). cFos immunoreactivity is enhanced with biotin amplification. Journal of Histochemistry & Cytochemistry **42**: 1635-1642.

Bobrow, M. N., T. D. Harris, K. J. Shaughnessy and G. J. Litt (1989). Catalyzed reporter deposition, a novel method of signal amplification - application to immunoassays. Journal of Immunological Methods **125**(1-2): 279-285.

Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Joergensen, U. Witte and O. Pfannkuche (2000). "A marine microbial consortium apparently mediating anaerobic oxidation of methane." Nature **407**(6804): 623-626.

Borneman, J. (1999). Culture-independent identification of microorganisms that respond to specified stimuli. Applied & Environmental Microbiology **65**(8): 3398-

Boye, M., A. A. Feenstra, C. Tegtmeier, L. O. Andresen, S. R. Rasmussen and V. Bille-Hansen (2000). Detection of Streptococcus suis by in situ hybridization, indirect immunofluorescence, and peroxidase-antiperoxidase assays in formalin-fixed, paraffin-embedded tissue sections from pigs. Journal of Veterinary Diagnostic Investigation **12**(3): 224-232.

Boylen, C. W. and J. C. Ensign (1970). Intracellular substrates for endogenous metabolism during long-term starvation of rod and spherical cells of *Arthrobacter crystallopoietes*. Journal of Bacteriology **103**: 578-587.

Brock, T. D. (1967). Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science **155**: 81-83.

Burleigh, I. G. and E. A. Dawes (1967). Studies on the endogenous metabolism and senescence of starved *Sarcina lutea*. Biochemistry Journal **102**: 236-250.

Cangelosi, G. A. and W. H. Brabant (1997). Depletion of Pre-16S rRNA in starved *Escherichia coli* cells. Journal of Bacteriology **179**: 4457-4463.

Chao, J., R. DeBiasio, Z. Zhu, K. A. Giuliano and B. F. Schmidt (1996). Immuno-fluorescence signal amplification by the enzyme-catalyzed deposition of a fluorescent reporter substrate (CARD). Cytometry **23**: 48-53.

Choi, J. W., E. B. Sherr and B. F. Sherr (1996). Relation between presence-absence of a visible nucleoid and metabolic activity in bacterioplankton cells. Limnology & Oceanography **41**(6): 1161-1168.

Cottrell, M. T. and D. L. Kirchman (2000). Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Applied & Environmental Microbiology **66**: 1692-1697.

Davis, B. D., S. M. Luger and P. C. Tai (1986). Role of ribosome degradation in the death of starved *Escherichia coli* cells. Journal of Bacteriology **166**: 439-445.

Dawes, E. A. and D. W. Ribbons (1965). Studies on the endogenous metabolism of *Escherichia coli*. Biochemistry Journal **95**: 332-343.

DeLong, E. F., L. T. Taylor, T. L. Marsh and C. M. Preston (1999). Visualization and enumeration of marine planktonic archaea and bacteria by using polyribo-nucleotide probes and fluorescent in situ hybridization. Applied & Environmental Microbiology **65**(12): 5554-5563.

DeLong, E. F., G. S. Wickham and N. R. Pace (1989). Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. Science **243**: 1360-1363.

DeLong, E. F., K. Y. Wu, B. B. Prezelin and R. V. M. Jovine (1994). High abundance of *Archaea* in Antarctic marine picoplankton. Nature **371**: 695-697.

Dinjens, W. N. M., J. Tenkate, M. Lenders, E. P. M. Vanderlinden and F. T. Bosman (1992). Bromodeoxyuridine (BrdU) Immunocytochemistry By Exonuclease-III (Exo-III) Digestion. Histochemistry **98**(3): 199-205.

Dirks, R. W. (1996). RNA molecules lighting up under the microscope. Histochemistry & Cell Biology **106**: 151-166.

Ducklow, H. W. (1999). The bacterial component of the oceanic euphotic zone. FEMS Microbiology Ecology **30**(1): 1-10.

Eilers, H., J. Pernthaler and R. Amann (2000a). Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. Applied & Environmental Microbiology **66**(11): 4634-4640.

Eilers, H., J. Pernthaler, F. O. Glöckner and R. Amann (2000b). Culturability and in situ abundance of pelagic bacteria from the North Sea. Applied & Environmental Microbiology **66**(7): 3044-3051.

Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts and R. Amann (2001). Isolation of novel pelagic bacteria from the German bight and their seasonal contributions to surface picoplankton. Applied & Environmental Microbiology **67**(11): 5134-5142.

Fegatella, F., J. Lim, S. Kjelleberg and R. Cavicchioli (1998). Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium Sphingomonas sp. strain RB2256. Applied & Environmental Microbiology **64**(11): 4433-4438.

Flärdh, K., P. S. Cohen and S. Kjelleberg (1992). Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. Journal of Bacteriology **174**(21): 6780-6788.

Freter, R., H. Brickner, M. M. Vickerman and K. E. Carey (1983). Survival and implantation of *Escherichia coli* in the intestinal tract. Infection & Immunity **39**: 686-

Fuchs, B. M., F. O. Glöckner, J. Wulf and R. Amann (2000). Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. Applied & Environmental Microbiology **66**(8): 3603-3607.

Fuhrman, J. A. and F. Azam (1982). Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: Evaluation and field results. Marine Biology **66**: 109-120.

Fuhrman, J. A. and A. A. Davis (1997). Widespread Archaea and novel bacteria from the deep sea as shown by 16S rRNA gene sequences. Marine Ecology Progress Series **150**(1-3): 275-285.

Fuhrman, J. A., K. McCallum and A. A. Davis (1992). Novel major archaebacterial group from marine plankton. Nature **356**(6365): 148-149.

Fuhrman, J. A. and C. C. Ouverney (1998). Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native *Archaea* with fluorescent single cell probes. Aquatic Ecology **32**: 3-15.

Fuhrman, J. A., T. D. Sleeter, C. A. Carlson and L. M. Proctor (1989). Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. Marine Ecology Progress Series **57**: 207-217.

Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman and A. Hagström (1999). Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Applied & Environmental Microbiology **65**(10): 4475-4483.

Gausing, K. (1977). Regulation of ribosome production in *Escherichia coli*: Synthesis and stability of ribosomal RNA and of ribosomal protein messenger RNA at different growth rates. Journal of Molecular Biology **115**: 35-354.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer and K. G. Field (1990). Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**: 60-63.

Givskov, M., L. Eberl, S. Möller, L. K. Poulsen and S. Molin (1994). Responses to Nutrient Starvation in Pseudomonas-Putida Kt2442 - Analysis of General Cross-Protection, Cell-Shape, and Macromolecular Content. Journal of Bacteriology **176**(1): 7-14.

Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius and K. H. Schleifer (1996). An in situ hybridization protocol for detection and identification of planktonic bacteria. Systematic & Applied Microbiology **19**(3): 403-406.

Glöckner, F. O., B. M. Fuchs and R. Amann (1999). Bacterioplankton composition in lakes and oceans: a first comparison based on fluorescence in situ hybridization. Applied & Environmental Microbiology **65**(8): 3721-3726.

Glöckner, F. O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Pernthaler and R. Amann (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Applied & Environmental Microbiology **66**(11): 5053-

Gourse, R. L., H. A. de Boer and M. Nomura (1986). DNA determinants of rRNA synthesis in E. coli: growth rate dependent regulation, feedback inhibition, upstream activation, antitermination. Cell **44**: 197-205.

Harper, S. J., J. H. Pringle, A. Gillies, A. C. Allen, L. Layward, J. Feehally and I. Lauder (1992). Simultaneous in situ hybridization of native mRNA and immunoglobulin detection by conventional immunofluorescence in paraffin wax embedded sections. Journal of Clinical Pathology **45**: 114-121.

Heissenberger, A., G. G. Leoppard and G. J. Herndl (1996). Relationship between the intracellular integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. Applied & Environmental Microbiology **62**(12): 4521-4528.

Heppelmann, B., R. Senaris and P. C. Emson (1994). Combination of alkaline phosphatase in situ hybridization with immunohistochemistry: colocalization of calretinin-mRNA with calbindine and tyrosine hadroxylase immunoreactivity in rat substantia nigra neurons. Brain Research **635**: 293-299.

Hobbie, J. E., R. J. Daley and S. Jasper (1977). Use of nuclepore filters for counting bacteria by fluorescence microscopy. Applied & Environmental Microbiology **33**(5): 1225-8.

Hopman, A. H. N., M. Kamps, J. Dederen, E. J. M. Speel and F. C. S. Ramekers (1998). Bicolor ISH using CARD amplification for the rapid and efficient analysis of chromosomal imbalances in cancer. Cytometry Supply **9**: 146-147.

Horiuchi, T. (1959). RNA degradation and DNA AND protein synthesis of *E. coli* in a phosphate deficient culture cycle. Journal of Biochemistry **46**: 1467-1480.

Jannasch, H. W. and G. E. Jones (1959). Bacterial populations in sea water as determined by different methods of enumeration. Limnology & Oceanography **4**: 128-139.

Juretschko, S., W. Schönhuber, S. Kulakauskas, D. Ehrlich, K. Schleifer and R. Amann (1999). In situ detection of Escherichia coli cells containing ColE1 related plasmids by hybridization to regulatory RNA II. Systematic & Applied Microbiology **22**(1): 1-8.

Karl, D. M. (1986). Determination of in situ microbial biomass, viability, metabolism, and growth. Bacteria in Nature. J. S. Poindexter and E. R. Leadbetter. New York , London, Plenum Press. **2:** 85-176.

Karner, M., E. F. DeLong and D. M. Karl (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409**: 507-509.

Karner, M. and J. A. Fuhrman (1997). Determination of active marine bacterioplankton: A comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Applied & Environmental Microbiology **63**(4): 1208-1213.

Kemp, P. F., S. Lee and J. LaRoche (1993). Estimating the growth rate of slowly growing marine bacteria from RNA content. Applied & Environmental Microbiology **59**: 2594-2601.

Kerkhof, L. and P. Kemp (1999). Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth. FEMS Microbiology Ecology.

Kirchman, D., H. Ducklow and R. Mitchell (1982). Estimates of bacterial growth from changes in uptake rates and biomass. Applied & Environmental Microbiology **44**(6): 1296-1307.

Kirchman, D. L. (1993). Leucine incorporation as a measure of biomass production by heterotrophic bacteria. Handbook of methods in aquatic microbial ecology. P. F. Kemp, B. F. Sherr, S. E.B. and J. J. Cole. Florida, Lewis Publishers**:** 509-512.

Kjelleberg, S. (1993). Starvation in Bacteria. New York, Plenum Press.

Larsson, L.-I. and D. M. Hougaard (1991). Combined non-radioactive detection of peptide hormones and their mRNAs in endocrine cells. Histochemistry **96**: 375-380.

Lebaron, P., P. Catala, C. Fajon, F. Joux, J. Baudart and L. Bernard (1997). A new sensitive, whole-cell hybridization technique for detection of bacteria involving a biotinylated oligonucleotide probe targeting rRNA and tyramide signal amplification. Applied & Environmental Microbiology **63**(8): 3274-3278.

Lee, N., P. Nielsen, K. Andreasen, S. Juretschko, J. Nielsen, K. Schleifer and M. Wagner (1999). Combination of fluorescent in situ hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. Applied & Environmental Microbiology **65**(3): 1289-1297.

Lee, S. H., C. Malone and P. F. Kemp (1993). Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure celluar RNA from natural planktonic bacteria. Marine Ecology Progress Series **101**: 193-201.

Licht, T. R., T. Tolker-Nielsen, K. Holmstrøm, K. A. Krogfelt, J. Urbance and S. Molin (1999). Inhibition of *Escherichia coli* precursor-16S rRNA processing by mouse intestinal contents. Environmental Microbiology **1**: 23-32.

Loretz, C. A. (1995). Electrophysiology of ion transport in teleost intestinal cells. Cellular and Molecular Approaches to Fish Ionic Regulation. C. M. Wood and T. J. Shuttleworth. Toronto, Academic Press**:** 25-56.

Ludwig, W., S. Dorn, N. Springer, G. Kirchhof and K. H. Schleifer (1994). PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Applied & Environmental Microbiology **60**(9): 3234-44.

Maaloee, O. and N. O. Kjeldgaard (1966). Control of macromolecular synthesis. New York, W.A. Benjamin.

Macecheko, P.T., L. Krueger, B. Hirsch, S.L. Erlandsen (1997). Comparison of immunologic amplification vs enzymatic deposition of fluorochrome-conjugated tyramide as detection systems for FISH. Journal of Histochemistry & Cytochemistry **45**: 359-363

Mayer, G. and M. Bendayan (1999). Immunogold signal amplification: application of the CARD approach to electron microscopy. Journal of Histochemistry & Cytochemistry **47**: 421-429.

Medveczky, N. and H. Rosenberg (1971). Phosphate transport in *Escherichia coli.* Biochimica & Biophysica Acta **241**: 494-506.

Merz, H., R. Malisius, S. Mannweiler, R. Zhou, W. Hartmann, K. Orscheschek, P. Moubayed and A. C. Feller (1995). Immunomax. A maximized immunohistochemical method for the retrieval and enhancement of hidden antigens. Lab Invest. **73**: 149-156.

Molin, S. and M. Givskov (1999). Application of molecular tools for in situ monitoring of bacterial growth activity. Environmental Microbiology **1**(5): 383-391.

Moran, R., Z. Darzykiewicz, L. Staiano-Coico and M. R. Melamed (1985). Detection of 5-bromodeoxyuridine (BrdUrd) incorporation by monoclonal antibodies: role of the denaturation step. Journal of Histochemistry & Cytochemistry **33**: 821-827.

Morita, R. Y. (1997). Bacteria in oligotrophic environments: starvation-survival lifestyle. New York, Chapman Hall.

Murray, A. E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge and E. F. DeLong (1999). A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. Aquatic Microbial Ecology **20**(2): 129-145.

Nazly, N., I. S. Carter and C. J. Knowles (1980). Adenine nucleotide pools during starvation of *Beneckea natriegens*. Journal of Genetical Microbiology **116**: 295-303.

Oda, Y., S. J. Slagman, W. G. Meijer, L. J. Forney and J. C. Gottschal (2000). Influence of growth rate and starvation on fluorescent in situ hybridization of *Rhodopseudomonas palustris*. FEMS Microbiology Ecology **32**(3): 205-213.

Odinot, P.T., J.F.G.M. Meis, J.A.A. Hoogkamp-Korstanje, W.J.G. Melchers (1998). In situ localization of *Yersinia enterocolitica* by catalyzed reporter deposition signal amplification. Journal of Clinical Pathology **51**: 444-449.

Oerther, D. B., J. Pernthaler, A. Schramm, R. Amann and L. Raskin (2000). Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Applied & Environmental Microbiology(66): 5.

Ouverney, C. C. and J. A. Fuhrman (1997). Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Applied & Environmental Microbiology **63**(7): 2735-2740.

Ouverney, C. C. and J. A. Fuhrman (2001). Marine planktonic archaea take up amino acids. Applied & Environmental Microbiology **67**(2): 1023-1023.

Pedros-Alio, C. and S. Y. Newell (1989). Microautoradiographic study of thymidine uptake in brackish waters around Sapelo Island, Georgia, USA. Marine Ecology Progress Series **55**: 83-94.

Plenat, F., E. Picard, L. Antunes, J-M. Vignaud, B. Marie, P. Chalabreysse, F. Muhale (1997). L'amplification des réactions immunologiques par dépot catalytique aux sites de réaction de dérivés de la tyramine. Annual Pathology **17**: 17-23

Poddighe, P.J., J. Bulten, H.M.J. Kerstens, J.C.M. Robben, W.J.G. Melchers, A.G.J.M. Hanselaar (1996). Human papilloma virus detection by in situ hybridization and signal amplification based on biotynilated tyramine deposition. Clinical Molecular Pathology **49**: 340-344

Pomeroy, L. R. (1974). The Ocean's Food Web, A Changing Paradigm. BioScience **24**(9): 499-504.

Porter, K. G. and Y. S. Feig (1980). The use of DAPI for identifying and counting aquatic microflora. Limnology & Oceanography **25**(5): 943-948.

Postgate, J. K. and J. R. Hunter (1962). The survival of starved bacteria. Journal of Genetical Microbiology **29**: 233-263.

Poulsen, L. K., G. Ballard and D. A. Stahl (1993). Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Applied & Environmental Microbiology **59**(5): 1354-1360.

Poulsen, L. K., F. Lan, C. S. Kristensen, P. Hobolth, S. Molin and K. A. Krogfelt (1994). Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA in situ hybridization. Infection & Immunity **62**(11): 5191-4.

Rizzoli, R., N. M. Maraldi, A. Galanzi, N. Zini, M. Falconi, M. Vitale and G. Mazzotti (1988). High-sensitivity detection of DNA-synthesis by immunolocalization of bromodeoxyuridine. Institute of Physics Conference Series(93): 551-552.

Sanno, N., A. Teramoto, M. Sugiyama, Y. Itoh and R. Y. Osamura (1996). Application of catalyzed signal amplification in immunodetection of gonadotropin subunits in clinically nonfunctioning pituitrary adenomas. American Journal of Clinical Pathology **106**: 16-21.

Schaechter, M., O. Maaloe and N. O. Kjeldgaard (1958). Dependency on medium and temperature of cell size and chemical compostion during balanced growth of *Salmonella typhimurium*. Journal of General Microbiology **19**: 592-606.

Scherer, C. G. and C. W. Boylen (1977). Macromolecular synthesis and degradation in *Arthrobacter* during periods of nutrient deprivation. Journal of Bacteriology **132**: 548-589.

Schönhuber, W., B. Fuchs, S. Juretschko and R. Amann (1997). Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Applied & Environmental Microbiology **63**(8): 3268-3273.

Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig and R. Amann (1999). In situ identification of cyanobacteria with horseraddish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Applied & Environmental Microbiology **65**(3): 1259-1267.

Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins and D. K. Button (1993). Isolation of typical marine bacteria by dilution culture growth maintenance and characteristics of isolates under laboratory conditions. Applied & Environmental Microbiology **59**(7): 2150-2160.

Shapiro, H. M. (1995). Practical Flow Cytometry. New York, Wiley-Liss, Inc.

Sherr, B., E. Sherr and P. del Giorgio (2001). Enumeration of total and highly active bacteria. Methods in Microbiology, Vol 30. **30:** 129-159.

Sherr, B. F., P. Del Giorgio and B. Sherr Evelyn (1999). Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. Aquatic Microbial Ecology **18**(2): 117-131.

Shi, S.-R., M. E. Key and K. L. Kalra (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. Journal of Histochemistry & Cytochemistry **39**: 741-748.

Simek, K. (1986). Bacterial Activity in a Reservoir Determined By Autoradiography and Its Relationship to Phytoplankton and Zooplankton. Internationale Revue der Gesamten Hydrobiologie **71**(5): 593-612.

Simon, M. and F. Azam (1989). Protein content and protein synthesis rates of planktonic marine bacteria. Marine Ecology Progress Series **51**: 201-213.

Simon, M. and C. Wunsch (1998). Temperature control of bacterioplankton growth in a temperate large lake. Aquatic Microbial Ecology **16**(2): 119-130.

Speel, E. J. M., F. C. S. Ramaekers and A. H. N. Hopman (1995). Cytochemical detection systems for in-situ hybridization, and the combination with immunocytochemistry - Who is still afraid of red, green and blue? Histochemical Journal **27**(11): 833-858.

Speel, E. J. M., F. C. S. Ramaekers and A. H. N. Hopman (1997a). Sensitive multicolor fluorescence in situ hybridization using catalyzed reporter deposition (CARD) amplification. Journal of Histochemistry & Cytochemistry **45**(10): 1439-1446.

Speel, E. J. M., P. Saremaslani, P. Komminoth and A. H. N. Hopman (1997b). CARD signal amplification: An efficient method to increase the sensitivity of DNA and mRNA in situ hydridization. American Journal of Pathology **151**(5).

Speel, E.J.M., A.H.N. Hopman , P. Komminoth (2000). Signal amplification for DNA and mRNA in situ hybridization. In: Methods in molecular biology. Totowa, NJ, Humana Press

Steward, G. F. and F. Azam (1999). Bromodeoxyuridine as an alternative to H-3-thymidine for measuring bacterial productivity in aquatic samples. Aquatic Microbial Ecology. **19**(1): 57-66.

Strange, R. E., F. A. Dark and A. G. Ness (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. Journal of General Microbiology **25**: 61-76.

Takagi, S., M. L. McFadden, R. E. Humphreys, B. A. Woda and T. Sairenji (1993). Detection of 5-bromo-2-deoxyuridine (Brdurd) incorporation with monoclonal anti-Brdurd antibody after deoxyribonuclease treatment. Cytometry **14**(6): 640-648.

Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger and K.-H. Schleifer (1994). Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Applied & Environmental Microbiology **60**(9): 3228-3235.

Tuomi, P., K. M. Fagerbakke, G. Bratbak and M. Heldal (1995). Nutritional Enrichment of a microbial community - the effects on activity, elemental composition, community structure and virus production. FEMS Microbiology Ecology **16**(2): 123-134.

Urbach, E., K. L. Vergin and S. J. Giovannoni (1999). Immunochemical detection and isolation of DNA from metabolically active bacteria. Applied & Environmental Microbiology. **65**(3): 1207-1213.

Van Gijlswijk, R. P. M., H. J. M. A. A. Zijlmans, J. Wiegant, M. N. Bobrow, T. J. Erickson, K. E. Adler, H. J. Tanke and A. K. Raap (1997). Fluorochrome-labeled tyramides: use in immuno-cytochemistry and fluorescence in situ hybridization. Journal of Histochemistry & Cytochemistry **45**: 375-382.

Vanheusden, J., P. deJong, F. Ramaekers, H. Bruwiere, M. Borgers and G. Smets (1997). Fluorescein-labeled tyramide strongly enhances the detection of low bromodeoxyuridine incorporation levels. Journal of Histochemistry & Cytochemistry **45**(2): 315-319.

Wada, A., K. Igarashi, S. Yoshimura, S. Aimoto and A. Ishihama (1995). Ribosome modulation factor: Stationary phase-specific inhibitor of ribosome functions from *Escherichia coli*. Biochem. Biophys. Res. Commun. **214**: 410-417.

Wagner, M., M. Schmid, S. Juretschko, K. H. Trebesius, A. Bubert, W. Goebel and K. H. Schleifer (1998). In situ detection of a virulence factor mRNA and 16s rRNA in *Listeria monocytogenes*. FEMS Microbiology Letters **160**(1): 159-168.

Wallner, G., R. Amann and W. Beisker (1993). Optimizing fluorescent *in situ*-hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry **14**(2): 136-143.

Wilson, D. M. and C. Bianchi (1999). Improved immunodetection of nuclear antigens after sodium dodecyl sulfate treatment of formaldehyde-fixed cells. Journal of Histochemistry & Cytochemistry **47**(8): 1095-1100.

Yin, B., D. Crowley, G. Sparovek, J. D. Wanderley and J. Borneman (2000). Bacterial functional redundancy along a soil reclamation gradient. Applied & Environmental Microbiology **66**(10): 4361-4365.

Zaitsu, K. and Y. Ohkura (1980). New fluorogenic substrates for horseradish-peroxidase - rapid and sensitive assays for hydrogen-peroxide and the peroxidase. Analytical Biochemistry **109**(1): 109-113.

Zarda, B. (1994). Gold-, enzym- und fluoreszenzmarkierte Nukleinsäuresonden zur

licht- und elektronenmikroskopischen *in situ*-Identifizierung von Mikroorganismen. Fakultät für Chemie, Biologie und Geowissenschaften. München, Technische Universität München: 108.

Zubkov, M. V., B. M. Fuchs, S. D. Archer, R. P. Kiene, R. Amann and P. H. Burkill (2001a). Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. Environmental Microbiology **3**(5): 304-311.

Zubkov, M. V., B. M. Fuchs, P. H. Burkill and R. Amann (2001b). Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Applied & Environmental Microbiology **67**(11): 5210-5218.

Zweifel, U. L. and A. Hagström (1995). Total counts of marine *Bacteria* include a large fraction of non-nucleoid-containing *Bacteria* (Ghosts). Applied & Environmental Microbiology **61**(6): 2180-2185.

Teil II

Publikationen

Erläuterungen zu den Publikationen

Diese Dissertation basiert zu einem großen Teil auf vier Veröffentlichungen. Die Beiträge der verschiedenen Autoren werden im Folgenden erläutert.

1. Annelie Pernthaler, Jakob Pernthaler, Heike Eilers, Rudolf Amann. Growth patterns of two marine isolates: Adaptations to substrate patchiness? Applied & Environmental Microbiology. **67**(9):4077-4083, 2001 Sep.
   Entwicklung des Konzeptes von A.P. und J.P., Durchführung der Experimente A.P. und J.P., Entwicklung der quantitativen Fluoreszenz-in situ-Hybridisierung und Quantifizierung der rRNA Gehaltes A.P., Quantifizierung des Protein und DNA Gehaltes J.P., Bereitstellung der Isolate H.E., Erstellen des Manuskriptes von A.P. unter redaktioneller Mitarbeit von J.P. und R.A.

2. Annelie Pernthaler, Christina M. Preston, Jakob Pernthaler, Edward F. DeLong, Rudolf Amann. A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Applied & Environmental Microbiology. **68**(2):661-667, 2002 Feb.
   Entwicklung des Konzeptes durch alle Koautoren, Durchführung der Experimente A.P., C.M. und J.P., Beprobung von C.P., Synthese der Polynukleotidsonden C.P. und A.P., Datenerhebung von A.P.mit Ausnahme der Helligkeitsmessungen (J.P), Erstellen des Manuskriptes von A.P. unter redaktioneller Mitarbeit von J.P., C.P., R.A und E.DeL.

3. Annelie Pernthaler, Jakob Pernthaler, Rudolf Amann. Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria. Applied & Environmental Microbiology. In press.
   Entwicklung des Konzeptes von A.P. und J.P., Beprobung von J.P., *Methodenentwicklung und Durchführung der Experimente A.P., Erstellen des Manuskriptes von A.P. unter redaktioneller Mitarbeit von J.P. und R.A.*

4. Annelie Pernthaler, Jakob Pernthaler, Martha Schattenhofer, Rudolf Amann. Detection of DNA-synthesizing bacterial populations in coastal North Sea plankton. Submitted.
   Entwicklung des Konzeptes von A.P. und J.P., Beprobung von J.P., Methodenentwicklung von A.P., Durchführung der Experimente von A.P. mit Ausnahme der in situ Hybridisierungsdaten von SAR86, Roseobacter und Alteromonas (M.S.), Erstellen des Manuskriptes von A.P. unter redaktioneller Mitarbeit von J.P. und R.A.

1

Growth patterns of two marine isolates: Adaptations to substrate patchiness?

Annelie Pernthaler, Jakob Pernthaler, Heike Eilers, Rudolf Amann

Applied & Environmental Microbiology. **67**(9):4077-4083, 2001 Sep.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2001, p. 4077–4083 Vol. 67, No. 9
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.9.4077–4083.2001

Growth Patterns of Two Marine Isolates: Adaptations to Substrate Patchiness?

ANNELIE PERNTHALER, JAKOB PERNTHALER,* HEIKE EILERS, AND RUDOLF AMANN

Max-Planck-Institut für marine Mikrobiologie, D-28359 Bremen, Germany

Received 3 May 2001/Accepted 26 June 2001

During bottle incubations of heterotrophic marine picoplankton, some bacterial groups are conspicuously favored. In an earlier investigation bacteria of the genus *Pseudoalteromonas* rapidly multiplied in substrate-amended North Sea water, whereas the densities of *Oceanospirillum* changed little (H. Eilers, J. Pernthaler, and R. Amann, Appl. Environ. Microbiol. 66:4634–4640, 2000). We therefore studied the growth patterns of two isolates affiliating with *Pseudoalteromonas* and *Oceanospirillum* in batch culture. Upon substrate resupply, *Oceanospirillum* lagged threefold longer than *Pseudoalteromonas* but reached more than fivefold-higher final cell density and biomass. A second, mobile morphotype was present in the starved *Oceanospirillum* populations with distinctly greater cell size, DNA and protein content, and 16S rRNA concentration. Contrasting cellular ribosome concentrations during stationary phase suggested basic differences in the growth responses of the two strains to a patchy environment. Therefore, we exposed the strains to different modes of substrate addition. During cocultivation on a single batch of substrates, the final cell densities of *Oceanospirillum* were reduced three times as much as those *Pseudoalteromonas*, compared to growth yields in pure cultures. In contrast, the gradual addition of substrates to stationary-phase cocultures was clearly disadvantageous for the *Pseudoalteromonas* population. Different growth responses to substrate gradients could thus be another facet affecting the competition between marine bacteria and may help to explain community shifts observed during enrichments.

Prefiltration and confinement of marine bacterioplankton during enrichments (8, 43), dilutions (13), and enclosure experiments (36, 37) can result in changes of both taxonomic composition and phenotypic features of communities. The percentage of cells with higher per-cell rRNA, DNA, and protein content (8, 13, 15), the proportion of plate-countable cells (11), and the proportion of cells exhibiting higher metabolic activity (15, 41) have all been observed to increase. Often the original community is overgrown by a few genera of frequently cultured marine gamma-proteobacteria, e.g., *Vibrio* sp., *Alteromonas* sp., and *Pseudoalteromonas* sp. (8, 16, 37), which are, however, most probably not very abundant members of the bacterioplankton (8).

Are those microbes that are not enriched in bottles or enclosures in principle unable to grow on the offered substrates? The majority of pelagic bacteria and archaea are capable of incorporating mixes of radiolabeled amino acids (21, 29). In previous works, strains related to the genera *Roseobacter* (alpha-proteobacteria), *Oceanospirillum* sp. (gamma-proteobacteria), and *Cytophaga* sp. (*Bacteroidetes*), were isolated from North Sea water samples on a substrate mix of amino acids and monomers, yet members of these lineages were not enriched from North Sea plankton during incubations of filtrates on the same substrates (8, 9).

Bacterioplankton community change upon filtration and/or substrate addition may thus be a consequence of other features of the enriched populations, rather than of the ability to utilize a particular substrate. A considerable proportion of the substrates and bacterial productivity in coastal pelagic environments are distributed in microscale patches of variable concentration and size, such as algal "phycospheres," marine snow, or metazoan fecal pellets (3, 31). The particle-attached and free-living pelagic communities differ both in phenotypes and in taxonomic composition (1, 7). Individual microbial species or phylogenetic lineages within the bacterioplankton may consequently differ in their ability to succeed in habitats with steeper or flatter substrate gradients. We therefore hypothesized that bacteria which exhibit a more rapid growth response under batch culture "feast-and-famine" conditions (32) are also favored during enrichments of environmental samples.

Flow cytometry and image-analyzed epifluorescence microscopy are tools to study growth-related microbial cell features, e.g., size and macromolecular content, both in whole communities (13, 14, 25, 33, 45) and in individual populations (5, 20). For example, a high per-cell ribosome content is generally regarded as a feature of active bacteria in mixed assemblages (2). Pure culture studies show a dependence of total ribosome content on growth rate in continuous cultures (5, 22, 26, 34). Furthermore, it has been suggested that some bacteria maintain a high rRNA content (i.e., excess protein synthesis capacity) during nongrowth to be able to rapidly respond to changes in growth condition (10, 12). If this hypothesis is correct, bacterial strains that exhibit contrasting patterns of per-cell ribosome concentration during early stationary phase should also differ in their competition for more or less patchy substrates.

Batch growth experiments with two marine isolates were performed in pure culture and coculture on low concentrations of organic carbon. The selected strains are affiliated with gamma-proteobacterial genera that had exhibited contrasting responses during substrate-amended enrichments of environmental samples in an earlier study (8) (Table 1). Cell numbers and sizes and the patterns of rRNA, total nucleic acid, and protein content per cell were followed during the different

* 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. Abundances of *Pseudoalteromonas* sp. and *Oceanospirillum* sp. in enrichments of North Sea filtrates and FISH detectability of the two studied strains during long-term starvation (for details, see reference 8)

Species used	Abundances in enrichments (10^5 cells ml^{-1}) (mean ±SD) during incubation period of:			FISH detectability during starvation (% of total cells) (mean ± range) during incubation period of:		
	0 h	24 h	48 h	0 days	35 days	53 days
Pseudoalteromonas sp.	b.l.[a]	b.l.	3.28 (±0.40)	100 (±1.7)		71 (±28.4)
Oceanospirillum sp.[b]	0.06 (±0.2)	0.33 (±0.72)	0.44 (±0.32)	93 (±1.4)	4 (±0.6)	4 (±2.6)

[a] b.l., below limit for FISH counting (<1% total counts).
[b] *Oceanospirillum* sp. data from reference 8.

growth phases in pure cultures. The population sizes of the two strains were then monitored in cocultures to which substrates were either added in one batch or gradually.

MATERIALS AND METHODS

Batch cultures of single strains. Growth experiments were carried out on a synthetic medium previously used for ecophysiological investigations on a marine *Sphingomonas* sp. (39). A mix of monomers and amino acids as described by Eilers et al. (9) was added to the medium at micromolar concentrations. The two marine strains used in this study, *Oceanospirillum* sp. strain KT0923 and *Pseudoalteromonas* sp. strain KT0912.10 (45), were both isolated from surface waters in the German Bight of the North Sea (9). According to 16S rDNA gene sequence analysis, they are phylogenetically most closely affiliated with *Oceanospirillum commune* and *Pseudoalteromonas atlantica* (95.7 and 99.7% rDNA similarity, respectively). Prior to the experiments, both strains were maintained on liquid medium for several growth cycles. Six days after their last reinoculation, 4 liters of freshly prepared medium was inoculated at initial densities of approximately 10^5 cells ml^{-1}. Incubations were performed in two parallels at 15°C and with gentle stirring. Fifty-ml subsamples were taken at 30-min to 2-h intervals for the first 58 h and at longer intervals thereafter, were fixed for 30 min with formaldehyde solution (final concentration, 2% [vol/vol]), and were stored frozen (−80°C) until further processing.

For the competition experiments, strains were inoculated at densities of approximately 0.5×10^5 cells ml^{-1}. In one set of treatments ("batch cocultures"), substrates were present in the medium at the time of inoculation. In a second set ("extended batch cocultures"), portions of the substrate mix (1% of total) were added hourly to the medium by a peristaltic pump. In addition, two controls without substrates were inoculated with the two strains. Subsamples were aseptically taken at several time points and were treated as described above.

Flow cytometry. Samples were analyzed on a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, Calif.). Cell counts and DNA and protein quantifications were carried out as previously described by simultaneous staining with the fluorescent dyes HOECHST33342 and SYPRO (Molecular Probes, Leiden, The Netherlands) and by double excitation with UV and green lasers (265 and 543 nm) (27, 45). Fluorescence was measured with logarithmic signal amplification. All measurements were standardized to the fluorescence of latex beads (FluoroSpheres, yellow green, 2-μm diameter; Molecular Probes) added to each sample at known concentrations. Absolute bacterial abundances were determined from the ratios of beads to bacteria. Objects that showed both DNA and protein fluorescence above background levels were regarded as bacteria. At least 2,000 such positive events, excluding beads, were recorded per sample. To avoid errors due to clustering of cells, samples were sonicated for 5 s prior to measurements (OmniLab sonicator bath; Bandelin, Berlin, Germany). Depending on cell concentration, data were acquired for a few seconds to several minutes. Measurements were excluded from the evaluation of fluorescence intensities if a significant drift of signal during the acquisition period was detected. Analysis of samples from the first experimental vessel revealed instrument instabilities; therefore, DNA and protein fluorescence intensities were evaluated from samples of the second experimental vessel only. The relative number of events in the high- and low-DNA subpopulations was determined for time points when two separate maxima of DNA fluorescence were readily distinguishable in histogram plots. Within the DNA-rich cell fraction of *Oceanospirillum* sp. populations, the frequency of bacteria with a high or low protein content was quantified during lag phase.

FISH. Based on the flow cytometry counts, selected time points of the growth curves were analyzed by fluorescence in situ hybridization (FISH). Subsamples were filtered onto white membrane filters (GTTP, diameter, 47 cm; pore size, 0.2 μm; Millipore, Bedford, Mass.) and were hybridized with the CY3-labeled probe EUB338 (2) for quantitative FISH. Specific probes for *Pseudoalteromonas* sp. and *Oceanospirillum* sp. (9) were used to evaluate the competition experiment. Hybridization and washing buffers were composed as described previously (9, 17). To minimize differences between quantitative hybridizations, the handling time between incubation and washing was standardized. All filter sections from a complete time series were hybridized simultaneously in one single batch of hybridization buffer. Samples were air dried and embedded in VectaShield antifading mounting medium (Vector Laboratories, Burlingame, Calif.).

Image acquisition and analysis. Gray images of fluorescently labeled cells were acquired at ×100 magnification on a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany) (calibrated pixel length, 0.064 μm; 4,096 gray levels). Since the stability of a laser as excitation light source is superior to that of a mercury arc bulb, conditions of measurement setups are more readily reproduced. Probe fluorescence from excitation with a green laser (HeNe, 543 nm) was recorded at a scanning speed of 30 s. To ensure output stability, the laser was switched on at least 2 h prior to measurements. To minimize uncontrolled cell bleaching, microscopic focusing was carried out by rapid prescanning at low laser intensity rather than by illumination with the mercury arc bulb. Background fluorescence was excluded by appropriate adjustment of the pinhole, which was set to collect light from a 0.6-μm-thick optical section (corresponding to the average cell width). This optical sectioning, moreover, provided an efficient focusing aid during prescanning, as even small deviations from the optimal focal position resulted in a strong decrease of cell brightness. We avoided all microscopic fields in which brightness gradients of stained cells were apparent, because such gradients probably indicated that the respective filter positions were not sufficiently horizontal for brightness measurements within a 0.6-μm slice. A total of 300 to 1,000 individual cells from 10 to 20 images was analyzed per sample.

Images were processed and measured with the software MetaMorph (version 3.5; Universal Imaging, West Chester, Pa.). Object edges were established by Unsharp Masking (28). The gray image was smoothed by a 16- by 16-pixel square, low-pass kernel, downscaled to 95% of its original brightness, and subtracted from the original image. The resulting image was multiplied by 20, and noise was reduced by a 5- by 5-pixel neighborhood Median filter. The edge-enhanced images from a series were subsequently thresholded automatically at a preset intensity (gray value, 200 to 500). The binary image served as a mask for size and brightness detection. Edges were smoothed by morphological closing, and objects of <25 pixels and of >1,000 pixels were discarded. Each processed image was examined and if required was interactively edited prior to measurement (exclusion of irregularly shaped objects and separation of touching cells). Object area, perimeter, total, and mean gray values were recorded. Cell volumes were calculated from the measured area and perimeter (33). To compensate for potential differences between individual hybridization series, a sample from a time point with a low standard deviation of mean gray values (*Pseudoalteromonas* sp., parallel 2, 100 h) served as the internal standard. In each series of samples, this internal standard was also hybridized and evaluated and brightness values from different hybridization series were corrected accordingly.

RESULTS

Pure culture batch growth. Following transfer to fresh medium, the lag phase of *Pseudoalteromonas* sp. (defined as the period between inoculation and the first doubling of cell numbers) was significantly shorter (9 [±1] h) than that of *Oceanospirillum* sp. (25 [±1] h) (Fig. 1). The highest doubling times of *Pseudoalteromonas* sp. and *Oceanospirillum* sp. were 2.4 and 3.7 h, respectively. The *Pseudoalteromonas* sp. population ceased cell division after 27 (±1) h, *Oceanospirillum* sp. after

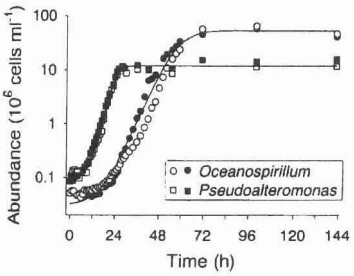


FIG. 1. Batch growth of *Oceanospirillum* sp. and *Pseudoalteromonas* sp. in two separate experiments.

100 h. At the onset of stationary phase, *Pseudoalteromonas* sp. had 18% of the cell density and 17% of the biomass of *Oceanospirillum* sp.

Relative 16S rRNA concentration per cell. Mean per-cell fluorescence after quantitative FISH with the 16S rRNA-targeted probe EUB338 was used to estimate changes in rRNA concentrations of *Oceanospirillum* sp. and *Pseudoalteromonas* sp. during growth (Fig. 2). Both organisms showed an increase of rRNA content before significant cell multiplication was detectable, and maximal RNA fluorescence intensity was approximately double its initial value in both strains. This maximum occurred during late logarithmic growth in *Oceanospirillum* sp. and at the onset of stationary phase in *Pseudoalteromonas* sp. The relative per-cell rRNA concentration of *Pseudoalteromonas* sp. was significantly elevated during 100 h of nongrowth. In

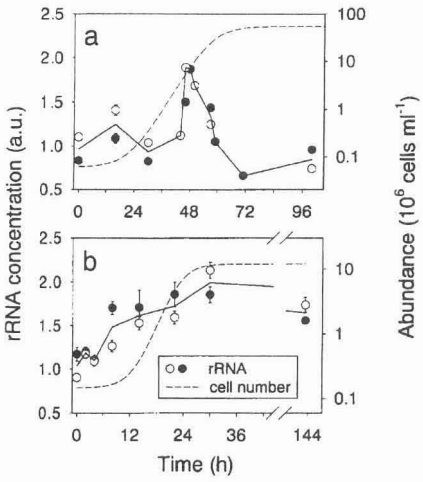


FIG. 2. Mean 16S rRNA concentration of *Oceanospirillum* sp. (a) and *Pseudoalteromonas* sp. (b) of two separate experiments (means ± 1 standard error). a.u., arbitrary units.

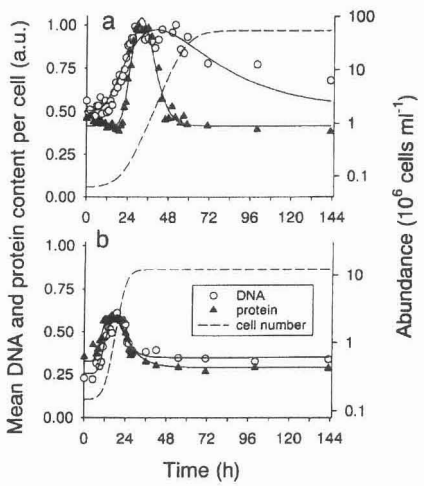


FIG. 3. Mean per-cell DNA and protein content of *Oceanospirillum* sp. (a) and *Pseudoalteromonas* sp. (b) during batch growth in pure cultures. a.u., arbitrary units.

contrast, rRNA fluorescence in *Oceanospirillum* sp. rapidly decreased to initial values at the onset of stationary phase.

DNA and protein fluorescence and cell sizes. Both organisms showed a bell-shaped curve of per-cell protein content during growth (Fig. 3). The relative protein fluorescence of *Oceanospirillum* sp. increased more rapidly than that of *Pseudoalteromonas* sp., to about 2.5 times of its initial value, whereas the maximum protein content of *Pseudoalteromonas* sp. was less than double its initial minimum. During stationary and exponential growth phases, the cellular protein content of *Oceanospirillum* sp. ranged from 75 (±15) fg cell^{-1} to 164 (±6) fg cell^{-1} and that of *Pseudoalteromonas* sp. ranged from 53 (±8) to 98 (±2) fg cell^{-1}, respectively. Maximum protein content per cell during mid-logarithmic growth corresponded with maximal cell volumes determined from size measurements of hybridized cells (data not shown). Mean per-cell DNA fluorescence intensity of both organisms approximately doubled during growth (Fig. 3). Except during mid-logarithmic growth, two subpopulations with different DNA content could be readily distinguished in both strains (Fig. 4). The high-DNA fraction represented about 25% in the *Oceanospirillum* sp. population even during stationary phase, whereas in *Pseudoalteromonas* sp., the high-DNA subpopulation declined to less than 5% in stationary-phase cells.

Population heterogeneity of *Oceanospirillum* sp. After 1 week of starvation and during lag phase, two distinct cell types were present in the *Oceanospirillum* sp. population: a small, nonmotile rod and a rare, large, fast-moving spirillum. The latter formed 1 to 2% of all cells at the time of inoculation and was not apparent during exponential growth or the first 24 h of stationary phase. FISH with a probe specific for *Oceanospirillum* sp. confirmed the purity of the culture (data not shown).

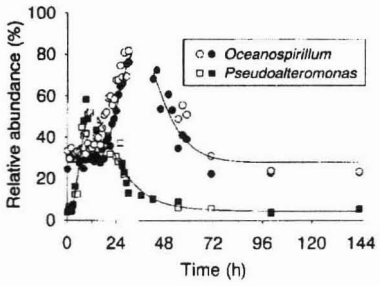


FIG. 4. Relative contribution of the fraction of cells with a high DNA content (multiple genome copies) in *Oceanospirillum* sp. and *Pseudoalteromonas* sp. The break in the curve indicates the time period during mid-logarithmic growth where a clear distinction of two DNA brightness classes was not possible.

The two subpopulations differed both in their cell sizes and mean rRNA fluorescence intensity, and the large size classes exhibited significantly higher rRNA concentrations at the end of lag phase (Fig. 5a) (analysis of variance, Scheffé post hoc comparisons, $P < 0.05$). No such subpopulations were observed in *Pseudoalteromonas* sp. (Fig. 5b). Two classes of cells with distinct protein content were distinguished in the DNA-rich subpopulation of *Oceanospirillum* sp. during lag phase (Fig. 6a to c) but not in *Pseudoalteromonas* sp. The *Oceanospirillum* sp. subpopulation of DNA-rich cells with distinctively higher protein fluorescence increased from <2% to 14% ± 2% after substrate addition and constituted >50% after the first doubling.

Competition between *Pseudoalteromonas* sp. and *Oceanospirillum* sp. Cocultures of the two strains always reached lower total cell densities ([5.8 ± 1.1] × 10^6 cells ml^{-1} [mean ± standard deviation]; $n = 6$) than the pure cultures of either strain. During coculture, *Oceanospirillum* sp. and *Pseudoalteromonas* sp. reached 7.5 and 25% of their pure culture maximum abundances, respectively (Fig. 7). The length of the lag phases and the duration of exponential growth of both organisms were similar in cocultures and in pure batch cultures. Thus, *Pseudoalteromonas* sp. had already ceased cell division at the onset of growth of *Oceanospirillum* sp. (Fig. 7). In both the batch and extended batch cocultures, where portions of substrates were added at intervals, *Oceanospirillum* sp. reached higher maximal cell densities than *Pseudoalteromonas* sp. During extended batch growth, *Pseudoalteromonas* sp. entered stationary phase when 30% of the total substrate had been added to the medium. It reached only 40% of the cell numbers attained in the batch cocultures (Fig. 7b). Total cell counts of *Oceanospirillum* sp. were similar after 100 h in both treatments. This resulted in three- to six-times-higher maximal densities of *Oceanospirillum* sp. than of *Pseudoalteromonas* sp. during extended batch cultivation, whereas the ratio of *Oceanospirillum* sp. to *Pseudoalteromonas* sp. was 1.3 in batch cocultures. No significant growth was observed in cocultures without substrate addition (data not shown).

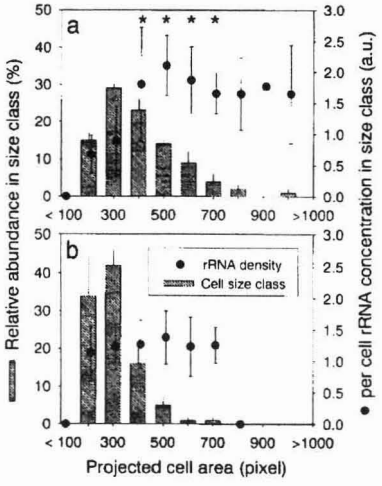


FIG. 5. Cell size distributions (bars) and distribution of mean 16S rRNA fluorescence (symbols) in different cell size classes (mean ± 1 standard deviation) at the end of lag phase of *Oceanospirillum* sp. (t = 16 h) (a) and of *Pseudoalteromonas* sp. (t = 8 h) (b). Asterisks indicate significant differences between the rRNA brightness of a size class and the brightness in the size classes of 100 to 200 and/or 200 to 300 pixels (analysis of variance, $P < 0.05$). a.u., arbitrary units.

DISCUSSION

Facultative eutrophic bacteria. Marine bacteria are frequently categorized into oligotrophic and eutrophic species. The latter are described as readily culturable, rare in bacterioplankton and prone to increase substantially in cell volume upon addition of substrate (40). The eutrophic bacterial strategy may represent the dominant type in some habitats, e.g., brackish waters (30), but common eutrophic isolates were generally rare in North Sea bacterioplankton (9). According to the above definition, both *Pseudoalteromonas* sp. and *Oceanospirillum* sp. are eutrophic marine genera.

Although frequently isolated, bacteria affiliating with the genus *Pseudoalteromonas* sp. were only occasionally detected on particles in coastal North Sea waters (8). High numbers of *Pseudoalteromonas*-specific viruses have been observed in fish feces (A. Wichels, personal communication), and a number of species from this genus are known to be associated with metazoans (18). Growth features that are commonly attributed to the opportunistic bacterial strategists were clearly more pronounced in the studied *Pseudoalteromonas* strain, such as the shorter lag phase upon transfer to fresh medium, a higher maximal growth rate, and lower total cell production (Fig. 1). It should be noticed, however, that all these parameters are potentially influenced by the composition of the cultivation medium. Thus, it would be premature to draw general conclusions about the ecological role of the two genera in North Sea coastal waters. Nevertheless, our results provide a model for

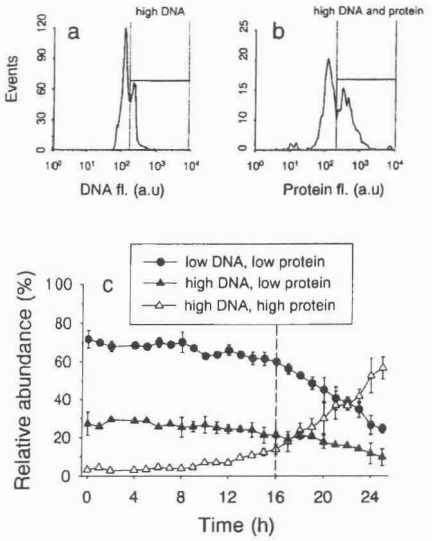


FIG. 6. (a) Histogram of bimodal distribution of DNA fluorescence (fl.) in *Oceanospirillum* sp. at *t* 16 h. (b) Histogram of bimodal protein fluorescence (fl.) within the high-DNA fraction of DNA fluorescence in panel a. (c) Relative abundances of subpopulations with different DNA and protein content in *Oceanospirillum* sp. during lag phase (0 to 16 h) and until the first doubling (25 h). a.u., arbitrary units.

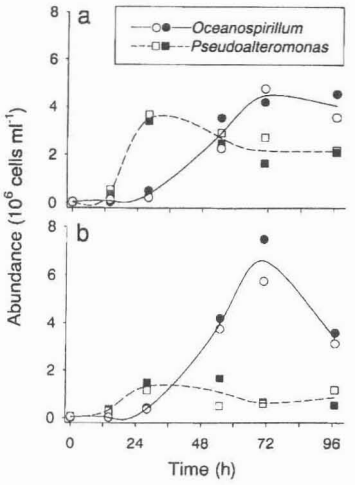


FIG. 7. Growth of *Oceanospirillum* sp. and *Pseudoalteromonas* sp. in cocultivation experiments. (a) Batch incubations. (b) Extended batch incubations with gradual substrate addition during 100 h.

understanding the outcome of our previous enrichment experiments on the same substrate mix (8) and illustrate the potential effects of substrate gradients on a two-species coculture system.

The stationary-phase subpopulation with a high DNA content in *Pseudoalteromonas* sp. was significantly smaller (Mann-Whitney U test, $P < 0.001$) than in *Oceanospirillum* sp., where it comprised roughly 25% of all cells (Fig. 4). Two other marine isolates also maintained large DNA-rich subpopulations in pure culture even during extended periods of starvation (20, 24). This contrasts somewhat with the view that the fraction of bacteria with a high DNA content found in pelagic microbial assemblages is representative of the growing part of the community (14, 25). Presently we can only speculate if and how the size of the high-DNA fraction during nongrowth is related to cultivation conditions or to the growth strategy of a population.

Marine spirilla have been known for several decades both from cultivation (44) and in situ observations (19). The phylogenetically closest relative of *Oceanospirillum* sp. strain KT0923, *O. commune*, was isolated from tropical surface waters (4). In coastal North Sea plankton, free-living bacteria related to *Oceanospirillum* sp. could be visualized in low densities (5×10^3 cells ml^{-1}) by FISH (8). The genus apparently includes culturable strains that are also present in the bacterioplankton and that are not oligotrophic by current definition (40).

A second phenotype was present in starved *Oceanospirillum* sp. cultures, which was clearly separated from the majority of cells by size, higher protein content, motility, and per-cell rRNA concentration (Fig. 5a and 6c). The rapid increase of such cells in stationary *Oceanospirillum* sp. after substrate addition (Fig. 6) suggests that cell multiplication mainly originated from within this subpopulation. Such heterogeneous growth has been observed before in marine bacteria. Upon substrate resupply, only a small fraction of a nongrowing *Vibrio* sp. population regained motility prior to cell multiplication (42). The starvation-induced motile subpopulation in *Oceanospirillum* sp. might thus be part of a more complex life strategy and, e.g., play a role in the colonization of new substrate patches (6).

Quantification of FISH staining intensities. Quantitative measurements of fluorescence intensities after FISH staining and image-analyzed microscopy yield two parameters as a potential measure of the 16S rRNA content per cell, the mean object gray value (optical brightness [O.B.]) and the total object gray value (integrated optical brightness [I.O.B.]). I.O.B. is the sum of fluorescence intensities of every positive pixel of a digitized image of a cell. The O.B. is the I.O.B. divided by the number of positive pixels, i.e., the mean pixel intensity.

The total amount of rRNA per cell that can be determined in chemical assays (22, 23), slot blot hybridizations (28), or flow cytometry evaluation of FISH-stained cells (5) is proportional to the sum of ribosomes per cell and is therefore equivalent to the I.O.B. of a hybridized cell. In batch culture studies, I.O.B. might be of limited use, because the bacterial cell volume substantially influences the total amount of ribosomes per cell. Thus, fluctuations in I.O.B. will to a large extent reflect changes in cell volume (35), even though the mean cell size and

I.O.B. are not expected to change completely in parallel during batch growth.

The O.B., on the other hand, is related to rRNA concentration, i.e., the density of ribosomes per unit of cell volume. The inherent advantage of the mean cell fluorescence as a measure of growth or protein synthesis potential is therefore its independence of changes in cell volume. It has been demonstrated that the cellular ribosome concentration (or its equivalent, the I.O.B. divided by the cell volume) increases with growth rate both in *Desulfovibrio vulgaris* and in *Pseudomonas putida* during balanced growth (26, 34).

The two bacterial strains studied clearly differed in their patterns of cellular 16S rRNA concentration during the various phases of their growth cycle (Fig. 5). During 100 h of stationary phase, high ribosome concentrations per cell were observed in *Pseudoalteromonas* sp. (Fig. 2b). Such maintenance of excess rRNA in a marine *Vibrio* sp. during starvation has been interpreted as an adaptation to a feast-and-famine existence, to allow rapid initiation of protein synthesis upon substrate resupply (12). The more rapid growth response of *Pseudoalteromonas* sp. both in pure culture and in cocultures (Fig. 7) and its selective enrichment in substrate-amended plankton samples (Table 1) provide evidence for this hypothesis. In contrast, the ribosome concentration of *Oceanospirillum* sp. declined upon the onset of stationary phase to the levels of the prestarved culture. The per-cell rRNA content of a *Sphingomonas* sp. that is thought to be representative of the free-living marine bacteria decreased by 90% upon cessation of growth (10). This development of cellular 16S rRNA concentrations during batch cultivation agrees with earlier findings that starvation periods of several weeks result in a much more pronounced decline of FISH detectability in cultures of *Oceanospirillum* sp. than in those of *Pseudoalteromonas* sp. (8) (Table 1).

We must, however, caution against overinterpretetion of the observed differences in ribosome content between the strains. A higher measurement frequency might be required to gain a detailed understanding of the actual development of cellular rRNA content during periods of rapid change, e.g., logarithmic growth. More studies are required to investigate other aspects which could potentially affect the patterns of rRNA concentration during batch growth. For example, it is presently unknown if and how the composition of the cultivation medium affects the patterns of macromolecular content. We used an artificial seawater mix that was specifically developed for the isolation of an oligocarbophilic marine *Sphingomonas* sp. and for subsequent ecophysiological investigations (38, 39), and this artificial seawater was successfully used for the isolation of the two studied strains. Yet this does not prove that the medium provided optimal growth conditions for the studied microbes.

Growth in cocultures. Numerous bacteria, including several *Pseudoalteromonas* species, are known to inhibit other microorganisms by releasing allelopathic substances (18). We found no indication for such interactions between the studied strains. Cell densities of *Oceanospirillum* sp. decreased during the first 24 h of nongrowth in the gradual enrichment, but no such decline was observed during batch cocultures, at higher total densities of *Pseudoalteromonas* sp. In contrast, mortality of stationary-phase *Pseudoalteromonas* sp. was higher in common batch culture enrichments. The lower abundances of both populations added together, compared to the density of either strain in pure culture (Fig. 1 and 7), rather indicated that cocultivation negatively affected the growth of both species.

Cocultivation and enrichment mode clearly influenced the growth rates and total cell production of the two species, but the duration of both the lag and of the respective exponential growth phases was unaffected by the treatments (Fig. 7). This may allow predictions about the performance of particular strains in batch coculture from parameters that can be readily determined in pure culture studies, provided that cocultivation is performed on the same medium.

From the length of the lag phases and the total cell production in pure cultures, it was predicted that the abundance ratio of the two strains in stationary-phase cocultures should be influenced by the mode of substrate addition. We hypothesized that *Pseudoalteromonas* sp. should dominate in a classic batch enrichment, whereas the more slowly but more "efficiently" growing *Oceanospirillum* sp. (Fig. 1) should be favored in a setup with gradually added substrates.

This was only partially verified. In batch cocultures the total cell production of *Oceanospirillum* sp. was indeed reduced to a much greater extent than that of *Pseudoalteromonas* sp., compared to pure cultures (Fig. 1 and 7a). The most obvious advantage of *Pseudoalteromonas* sp. under these conditions was the shorter growth delay upon substrate addition, in both pure and mixed cultures (Fig. 1 and 7). Therefore, *Pseudoalteromonas* sp. probably consumed the bulk of available organic matter. On the other hand, *Oceanospirillum* sp. was not only capable of growth on the fraction of substrate that was not consumed by *Pseudoalteromonas* sp.; it eventually even reached higher total densities than the other strain in batch coculture. This agrees with the higher total cell production of *Oceanospirillum* sp. in pure culture (Fig. 1).

In contrast, the shorter lag phase of *Pseudoalteromonas* sp. would represent no specific advantage during gradual substrate addition. The significantly reduced growth of *Pseudoalteromonas* sp. in extended batch cocultures (Mann-Whitney U test, $n = 8$, $P < 0.01$) (Fig. 7b) is therefore most likely the consequence of a lower amount of available substrate at the onset of cell multiplication. Less than 20% of the organic carbon of the batch culture had been added at that time point. The gradual addition of substrates to stationary cocultures of the two strains did not result in lower final abundances of *Oceanospirillum* sp., and the slopes of cell increase during exponential growth of *Oceanospirillum* sp. were unaffected or even slightly higher in the gradual enrichments (Fig. 7a and b). In summary, there is evidence for both strains that the mode of substrate addition affected competition between *Pseudoalteromonas* sp. and *Oceanospirillum* sp. in batch coculture.

Conclusions. Under our specific cultivation conditions, neither the length of lag phases of the studied strains nor the duration of logarithmic growth appeared to be affected by cocultivation. The selective enrichment of *Pseudoalteromonas* sp. on a particular substrate mix, as previously observed in pelagic samples (8), is therefore most likely related to a shorter growth delay upon addition of these substrates. The *Pseudoalteromonas* sp. strain, moreover, maintained high stationary-phase levels of cellular rRNA, which has been predicted for marine bacteria with a more opportunistic life strat-

egy. This hypothesis was supported by the outcome of gradual substrate addition to cocultures, which resulted in a shift of total cell production towards *Oceanospirillum* sp. Gradual enrichment might, therefore, provide a tool for the directed isolation of bacteria that are otherwise rapidly overgrown.

ACKNOWLEDGMENTS

We thank B. Fuchs for fruitful discussions on the topic of brightness measurements, B. MacGregor for critical reading of the manuscript, and N. Neese for advice on fluorescence staining.

This work was supported by the German Ministry of Education and Research (BMBF, project BIOLOG) and by the Max Planck Society.

REFERENCES

1. **Acinas, S. G., J. Antón, and F. Rodríguez-Valera.** 1999. Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Appl. Environ. Microbiol. **65:**514–522.
2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Azam, F.** 1998. Microbial control of oceanic carbon flux: the plot thickens. Science **280:**694–696.
4. **Baumann, L., P. Baumann, M. Mandel, and R. D. Allen.** 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. **110:**402–429.
5. **Binder, B. J., and Y. C. Liu.** 1998. Growth rate regulation of rRNA content of a marine *Synechococcus* (cyanobacterium) strain. Appl. Environ. Microbiol. **64:**3346–3351.
6. **Blackburn, N., T. Fenchel, and J. Mitchell.** 1998. Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science **282:**2254–2256.
7. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924–934.
8. **Eilers, H., J. Pernthaler, and R. Amann.** 2000. Succession of pelagic marine bacteria during enrichment: a close look on cultivation-induced shifts. Appl. Environ. Microbiol. **66:**4634–4640.
9. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
10. **Fegatella, F., J. Lim, S. Kjelleberg, and R. Cavicchioli.** 1998. Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256. Appl. Environ. Microbiol. **64:**4433–4438.
11. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47:**49–55.
12. **Flärdh, K., P. S. Cohen, and S. Kjelleberg.** 1992. Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **174:**6780–6788.
13. **Fuchs, B. M., M. V. Zubkov, K. Sahm, P. H. Burkill, and R. Amann.** 2000. Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. Environ. Microbiol. **2:**191–201.
14. **Gasol, J. M., and X. A. G. Morán.** 1999. Effects of filtration on bacterial activity and picoplankton community structure as assessed by flow cytometry. Aquat. Microb. Ecol. **16:**251–264.
15. **Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and Å. Hagström.** 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl. Environ. Microbiol. **65:**4475–4483.
16. **Giuliano, L., E. De Domenico, M. G. Höfle, and M. M. Yakimov.** 1999. Identification of culturable oligotrophic bacteria within naturally occurring bacterioplankton communities of the Ligurian Sea by 16S rRNA sequencing and probing. Microb. Ecol. **37:**77–85.
17. **Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K.-H. Schleifer.** 1996. An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst. Appl. Microbiol. **19:**403–406.
18. **Holmström, C., and S. Kjelleberg.** 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol. Ecol. **30:**285–293.
19. **Jannasch, H.** 1967. Enrichments of aquatic bacteria in continuous culture. Arch. Mikrobiol. **59:**165–173.
20. **Joux, F., P. Lebaron, and M. Troussellier.** 1997. Changes in cellular states of the marine bacterium *Deleya aquamarina* under starvation conditions. Appl. Environ. Microbiol. **63:**2686–2694.
21. **Karner, M., and J. A. Fuhrman.** 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl. Environ. Microbiol. **63:**1208–1213.
22. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594–2601.
23. **Kerkhof, L., and P. Kemp.** 1999. Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth. FEMS Microbiol. Ecol. **30:**253–260.
24. **Lebaron, P., and F. Joux.** 1994. Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater. Appl. Environ. Microbiol. **60:**4345–4350.
25. **Li, W. K., J. F. Jellet, and P. M. Dickie.** 1995. DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. Limnol. Oceanogr. **40:**1485–1495.
26. **Moeller, S., C. S. Kristensen, L. K. Poulsen, J. M. Carstensen, and S. Molin.** 1995. Bacterial growth on surfaces: automated image analysis for quantification of growth-related parameters. Appl. Environ. Microbiol. **61:**741–748.
27. **Monger, B. C., and M. R. Landry.** 1993. Flow cytometric analysis of marine bacteria with Hoechst 33342. Appl. Environ. Microbiol. **59:**905–911.
28. **Oerther, D. B., J. Pernthaler, A. Schramm, R. Amann, and L. Raskin.** 2000. Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Appl. Environ. Microbiol. **66:**2154–2165.
29. **Ouverney, C. C., and J. A. Fuhrman.** 2000. Marine planktonic Archaea take up amino acids. Appl. Environ. Microbiol. **66:**4829–4833.
30. **Pinhassi, J., and A. Hagström.** 2000. Seasonal succession in marine bacterioplankton. Aquat. Microb. Ecol. **21:**245–256.
31. **Ploug, H., H. P. Grossart, F. Azam, and B. B. Jorgensen.** 1999. Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: implications for the carbon cycle in the ocean. Mar. Ecol. Prog. Ser. **179:**1–11.
32. **Poindexter, J. S.** 1981. Oligotrophy: feast and famine existence. Adv. Microb. Ecol. **5:**63–89.
33. **Posch, T., J. Pernthaler, A. Alfreider, and R. Psenner.** 1997. Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. Appl. Environ. Microbiol. **63:**867–873.
34. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
35. **Ruimy, R., V. Breittmayer, V. Boivin, and R. Christen.** 1994. Assessment of the state of activity of individual bacterial cells by hybridization with a ribosomal RNA targeted fluorescent probe. FEMS Microbiol. Ecol. **15:**207–214.
36. **Schäfer, H., L. Bernard, C. Courties, P. Lebaron, P. Servais, R. Pukall, E. Stackebrandt, M. Troussellier, T. Guindulain, J. Vives-Rego, and G. Muyzer.** 2001. Microbial community dynamics in Mediterranean nutrient-enriched seawater. FEMS Microbiol. Ecol. **34:**243–253.
37. **Schäfer, H., P. Servais, and G. Muyzer.** 2000. Successional changes in the genetic diversity of a marine assemblage during confinement. Arch. Microbiol. **173:**138–145.
38. **Schut, F.** 1994. Ph. D. thesis. University of Groningen, Groningen, The Netherlands.
39. **Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture growth maintenance and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59:**2150–2160.
40. **Schut, F., R. A. Prins, and J. C. Gottschal.** 1997. Oligotrophy and pelagic marine bacteria: facts and fiction. Aquat. Microb. Ecol. **12:**177–202.
41. **Sherr, E. B., B. F. Sherr, and C. T. Sigmon.** 1999. Activity of marine bacteria under incubated and in situ conditions. Aquat. Microb. Ecol. **20:**213–223.
42. **Stretton, S., S. J. Danon, S. Kjelleberg, and A. E. Goodman.** 1997. Changes in cell morphology and motility in the marine *Vibrio* sp. strain S14 during conditions of starvation and recovery. FEMS Microbiol. Lett. **146:**23–29.
43. **Suzuki, M. T.** 1999. Effect of protistan bacterivory on costal bacterioplankton diversity. Aquat. Microb. Ecol. **20:**261–272.
44. **ZoBell, C. E.** 1946. Marine microbiology. A monograph on hydrobacteriology. Chronica Botanica Company, Waltham, Mass.
45. **Zubkov, M. V., B. M. Fuchs, H. Eilers, P. H. Burkill, and R. Amann.** 1999. Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. Appl. Environ. Microbiol. **65:**3251–3257.

2

Comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea

Annelie Pernthaler, Christina M. Preston, Jakob Pernthaler, Edward F. DeLong, Rudolf Amann.

Applied & Environmental Microbiology. **68**(2):661-667, 2002 Feb.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2002, p. 661–667 Vol. 68, No. 2
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.2.661–667.2002

Comparison of Fluorescently Labeled Oligonucleotide and Polynucleotide Probes for the Detection of Pelagic Marine Bacteria and Archaea

Annelie Pernthaler,[1] Christina M. Preston,[2] Jakob Pernthaler,[1]*
Edward F. DeLong,[2] and Rudolf Amann[1]

Max-Planck-Institute for Marine Microbiology, Bremen, Germany,[1] and Monterey Bay Aquarium Research Institute, Moss Landing, California[2]

Received 8 October 2001/Accepted 15 November 2001

We compared the detection of bacteria and archaea in the coastal North Sea and at Monterey Bay, Calif., after fluorescence in situ hybridization (FISH) either with rRNA-targeted oligonucleotide probes monolabeled with the cyanin dye Cy3 (oligoFISH) or with fluorescein-labeled polyribonucleotide probes (polyFISH). During an annual cycle in German Bight surface waters, the percentages of bacteria visualized by polyFISH (annual mean, 77% of total counts) were significantly higher than those detected by oligoFISH (53%). The fraction of total bacteria visualized by oligoFISH declined during winter, whereas cell numbers determined by polyFISH remained constant throughout the year. Depth profiles from Monterey Bay showed large differences in the fraction of bacterial cells visualized by polyFISH and oligoFISH in the deeper water layers irrespective of the season. Image-analyzed microscopy indicated that the superior detection of cells by polyFISH with fluorescein-labeled probes in bacterioplankton samples was less a consequence of higher absolute fluorescence intensities but was rather related to quasi-linear bleaching dynamics and to a higher signal-to-background ratio. The relative abundances of archaea in North Sea and Monterey Bay spring samples as determined by oligoFISH were on average higher than those determined by polyFISH. However, simultaneous hybridizations with oligonucleotide probes for bacteria and archaea suggested that the oligoFISH probe ARCH915 unspecifically stained a population of bacteria. Using either FISH technique, blooms of archaea were observed in North Sea surface waters during the spring and summer months. Marine group II archaea (*Euryarchaeota*) reached >30% of total picoplankton abundances, as determined by polyFISH. We suggest that studies of pelagic microbial community structure using oligoFISH with monolabeled probes should focus on environments that yield detections ≥70% of total cell counts, e.g., coastal surface waters during spring and summer.

Different staining techniques are available for the microscopic identification of bacteria and archaea in environmental samples (1, 8, 13, 20). Although in principle all of these methods aim at a similar goal, the elucidation of microbial community structure, each approach is currently limited to addressing a particular set of topics. For example, bacterial chromosomal painting and immunofluorescence staining require the prior cultivation of the target organism yet provide high specificity for the detection of individual bacterial strains in environmental samples (20, 43). In contrast, general questions about the abundances of different bacterial groups and of archaea in marine pelagic and sediment microbial communities can be answered more readily by fluorescence in situ hybridization (FISH) with rRNA-targeted oligo- and polynucleotide probes (8, 17, 22). Since it is still poorly understood which microbial lineages substantially contribute to the picoplankton, FISH techniques are an increasingly popular tool for basic ecological research in marine microbiology (5, 7, 11, 12, 18, 21, 29, 31, 38, 39).

Fluorescently labeled rRNA-targeted oligonucleotides are used to specifically stain different members of microbial communities. The specificity of the probes ranges from the phylotype to the kingdom, depending on the targeted region on the rRNA (4, 9). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (35, 37). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (26, 32, 36), but it may hamper the microscopic or flow cytometric counting of nongrowing or starving bacteria by FISH with monolabeled oligonucleotides (2, 27). Presently, only the cyanide dye Cy3 provides sufficient signal intensity to serve as fluorescent label for the direct microscopic visualization of microbial assemblages in the marine environment by FISH with monolabeled oligonucleotide probes (11, 17, 19, 31, 39).

Recently, poly(ribo)nucleotide probes to discriminate marine bacteria and uncultured marine archaea have been successfully applied in the open ocean (18). Such probes are RNA transcripts from PCR amplicons of 16S and 23S rRNA genes from either environmental DNA or fosmid clones (8). Numerous fluorescently labeled uridine molecules are incorporated into the polynucleotides during transcription, and the probes are subsequently hydrolyzed under controlled conditions to obtain approximately 100-mer fragments. The fluorescently labeled rRNA-targeted polynucleotide probes have been reported to yield higher signal intensities than oligonucleotide probes (23, 42) and may thus represent a better means of detecting microbes with a low ribosome content in oligotrophic habitats.

We compared the performance of the two FISH approaches

* 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.

in different water bodies and during different seasons, using oligonucleotide and polynucleotide probes targeting bacteria and marine group 1 (G1; crenarchaeota) and group 2 (G2; euryarcheota) archaea (8). In addition, image analysis was applied for evaluation of brightness parameters that were considered relevant for microscopic quantification irrespective of the fluorescent label, i.e., the signal-to-background ratios and the bleaching dynamics of FISH-stained cells in environmental samples.

MATERIALS AND METHODS

Study sites and sample preparation. Between January and December 1998, seawater was collected biweekly from a 1-m depth at the sampling site Helgoland Roads (54°9′N, 7°52′E) near the island of Helgoland, which is situated 23 miles offshore in the German Bay of the North Sea (12). Water from Monterey Bay was collected on 12 April and 16 November 2000 with a rosette sampler at a station 10.8 miles offshore near Moss Landing, Calif., with a total depth of 1,097 m. Samples were fixed with 3.7% (vol/vol) formaldehyde solution overnight at 4°C and filtered onto white polycarbonate membrane filters (diameter, 25 or 47 mm; Millipore, Bedford, Mass.). Monterey Bay samples were rinsed with a 2% NaCl (wt/vol)–ethanol (50% [vol/vol]) solution, and North Sea samples were rinsed with particle-free distilled water. Filters were air dried and stored at −20°C until further processing.

Synthesis of polynucleotide probes. Cloning of rRNA operons, preparation of DNA templates, and generation of fluorescently labeled polyribonucleotide probes were carried out as described previously (8). Picoplankton DNA extracts of samples from both the Monterey Bay and the German Bight of the North Sea were used separately as templates to generate bacterial polynucleotide probes for each region.

FISH with oligonucleotide probes (oligoFISH). Sections of filters were hybridized as described previously (16) with the Cy3-monolabeled probes EUB338 and ARCH915 (Interactiva, Ulm, Germany), which are targeted to bacteria and to archaea, respectively (3, 41). The air-dried samples were mounted in a mixture that contained 5.5 parts Citifluor AF1 (Citifluor Ltd., London, United Kingdom), 1 part Vecta Shield (Vector Laboratories, Burlingame, Calif.), and 0.5 parts phosphate-buffered saline; the mixture was amended with 4′,6′-diamidino-2-phenylindole (DAPI) (final concentration, 1 μM) for counterstaining. Filters were then evaluated on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) equipped with an HBO 100 W Hg vapor lamp, appropriate filter sets for Cy3 and DAPI fluorescence (34), and a 100× Plan Apochromat objective. Between 700 and 1,000 DAPI-stained objects were counted per sample. Counts of bacteria from the German Bight after FISH with probe EUB338 were taken from Eilers et al. (12). Bleaching dynamics were determined with both fluorescein isothiocyanate (fluorescein)- and CY3-labeled probes.

FISH with polynucleotide probes (polyFISH). Fluorescein multiply labeled probes targeted to marine pelagic crenarcheota (probe G1), euryarcheota (probe G2), or bacteria (probe EUBAC) were used for FISH counts of environmental samples, and both fluorescein- and CY3-labeled polynucleotide probes targeted to bacteria were synthesized for the determination of bleaching dynamics. For FISH, pieces of filter were placed face up onto glass slides. Only 19 of 22 samples of the time series from the German Bight were available for counts with EUBAC. Depending on their size, the filter sections were covered with 10 to 20 μl of hybridization solution containing 5 ng of hydrolyzed polynucleotide probe/μl. The hybridization solution was prepared as described previously (8). The filter was then covered by a coverslip and placed in a 50-ml plastic tube. A piece of filter paper was placed inside the tube to maintain humidity, and 5 ml of 5× SET (1× SET is 150 mM NaCl, 1 mM Na_2EDTA, 20 mM Tris-HCl [pH 7.8]) was added. Hybridizations were incubated for 4 h at 55°C (50% formamide; probe EUBAC) or 65°C (70% formamide; probes G1 and G2). After hybridizations, the samples were washed for 1 h at 55°C in washing buffer (50% formamide, 0.2× SET). For microscopic evaluation the filter sections were embedded in the above-described mounting mix and the percentage of FISH-stained objects was quantified at blue (fluorescein) and UV (DAPI) excitations (8).

Image acquisition and analysis. Hybridized filter sections were inspected on an Axioplan II Imaging microscope (Carl Zeiss) (100× Plan Apochromat lens). Images (resolution, 1,280 by 1,024 pixels, 12 bits $pixel^{-1}$) were captured with a black and white slow-scan digital camera with approximately constant quantum efficiency between 440 and 580 nm wavelength (ORCA; Hamamatsu, Hersching, Germany) linked to a personal computer. Pictures were recorded and processed with KS400 image analysis software (Carl Zeiss). Exposure times ranged between 100 and 500 ms, and identical exposure times were used for the analysis of parallel samples with Cy3- or fluorescein-labeled oligonucleotide or polynucleotide probes.

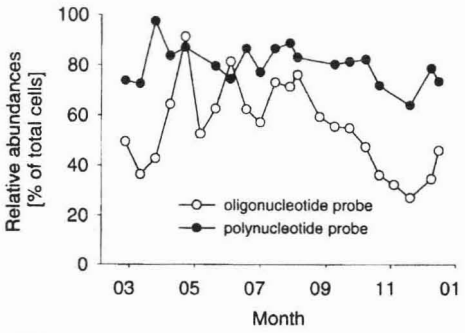


FIG. 1. Percentages of DAPI-stained cells in the German Bight of the North Sea (surface samples, 1998) detected by oligonucleotide (EUB338) and polynucleotide (EUBAC) probes targeted to bacteria. FISH data with the probe EUB338 are from reference 12.

Samples for the measurements of bleaching dynamics were prefiltered (1.2-μm pore size) and incubated for 24 h at in situ temperature in the dark prior to fixation, which typically results in a community shift towards larger gamma proteobacteria with a higher FISH signal intensity (10). For the bleaching time series, 50 images from one microscopic field were captured at intervals of 500 ms at blue (fluorescein) or green (Cy3) excitation and continuous illumination. To extend the observable bleaching period, the intensity of the 100 W mercury arc bulb was reduced by a diaphragm in the beam path; therefore, the acquired curves represent a minimum bleaching estimate. A binary mask image for cell detection was produced for the first image of a series (t_0), as described previously (28). The mask image was subsequently edited, and 10 randomly distributed background areas were added interactively. Changes in the mean grey levels of all positive objects in the t_0 image (between 50 and 200 cells per image) and of the background areas were then measured for the whole image series. Ten image series were averaged per sample, and the signal-to-background ratios were calculated as the quotient of mean object to mean background grey levels.

For a direct comparison of cells stained by either FISH technique, double images were acquired from the same microscopic fields at probe and DAPI excitation wavelengths. Ten randomly selected background areas were added to measurement masks interactively. Triplicate filter sections were analyzed per sample, and 700 cells were randomly chosen from 10 image pairs captured on each filter section. Image pairs were processed as described previously (33), but the evaluation strategy was modified as follows: probe-positive objects without DAPI signal were excluded from measurements. Measurement masks for objects that showed both probe and DAPI fluorescence ("positives") were produced from images of probe staining, whereas masks for all other DAPI-stained objects ("negatives") were deduced from DAPI staining. Such DAPI-positive objects without apparent probe signal were not used for the calculations of mean per cell fluorescence intensities. They are, however, included in Fig. 4, below, in order to depict the difference in signal-to-background ratios between probe-positive and probe-negative cells.

Statistical analysis. The nonparametric Wilcoxon matched pair test was used to analyze the percentages of total DAPI counts with either probe in North Sea samples. All other differences between two groups of data were tested for significance by using the Mann-Whitney rank sign test.

RESULTS AND DISCUSSION

Relative abundances of bacteria by oligoFISH and polyFISH. The percentages of DAPI counts of North Sea bacteria that could be visualized by polyFISH were significantly higher than those detected by oligoFISH ($n = 19$; $P < 0.01$) (Fig. 1). During the spring and summer months (April to September),

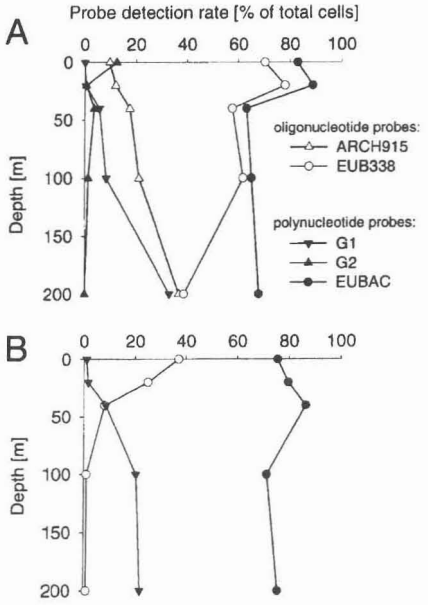


FIG. 2. Percentages of DAPI-stained cells in the upper 200 m of Monterey Bay detected with oligonucleotide and polynucleotide probes for bacteria and archaea, from 12 April 2000 (A) and 16 November 2000 (B). Oligonucleotide probes: ARCH915 (Archaea), EUB338 (bacteria). Polynucleotide probes: G1 (crenarchaeota), G2 (euryarchaeota), EUBAC (bacteria). Probes detecting <1% of DAPI-stained cells throughout the profiles are omitted from the graphs.

abundances determined by polyFISH were on average 10% higher and were less variable (mean, 78%; range, 64 to 87%) than by oligoFISH (66%; range, 52 to 91%) (12), but this difference was not statistically significant ($n = 9$; $P > 0.1$). Detection with probe EUB338 significantly decreased in autumn and winter (before 8 April and after 26 August) (mean, 43%; range, 27 to 55%) ($n_{summer} = 9$, $n_{winter} = 13$; $P < 0.01$), whereas detection with EUBAC remained high during this period (76%; range, 64 to 82%) and was statistically indistinguishable from the percentages of DAPI counts in spring and summer samples ($P > 0.05$). In the spring samples from Monterey Bay (Fig. 2A), the mean relative abundances of EUBAC and EUB338 in depths between 0 and 100 m was 75% (63 to 89%) and 67% (62 to 78%) of total cells, respectively. Less than 60% of EUBAC counts were visualized by EUB338 at 200 m depth. In Monterey Bay autumn samples, we could not detect any cells with oligoFISH below 50 m, whereas the polyFISH counts remained high in all depths (78%; range, 71 to 86%) (Fig. 2B). Test counts revealed no significant differences in the percentages of probe-stained cells between our laboratories with either technique. PolyFISH with probes prepared from DNA templates either from the North Sea or Monterey Bay resulted in equally high abundances in German Bight samples (data not shown).

Image analysis. FISH with Cy3-labeled oligonucleotide or polynucleotide probes clearly yielded higher initial signal-to-background ratios than that with probes labeled with fluorescein (Fig. 3). Although the magnitude of signal-to-background ratio and fluorescence bleaching is certainly related to sample preparation, optical properties of the equipment such as the age and power of the Hg vapor lamp, and to antibleaching agents in the mounting medium, the different dynamics of signal decrease of Cy3- and fluorescein-labeled probes were, nevertheless, striking. In spite of a microscopic embedding solution that had been developed and tested for its superior antifading properties, we observed a decrease in the signal-to-background ratio of bacteria stained with Cy3-labeled oligo- or polynucleotide probes of over 50% within the first 30 s of illumination (Fig. 3B). Initially, the signal-to-background ratios for bacteria stained with fluorescein-multilabeled polynucleotide probes was only marginally higher than that with Cy3-labeled oligonucleotide probes. After 30 s of illumination, the signal-to-background ratios for cells stained by polyFISH with fluorescein-labeled probes were still twice as high as that of cells after oligoFISH with Cy3-labeled probes. Since the counting of a microscopic field may require time periods of several seconds up to minutes, bleaching potentially represents an important feature of a staining procedure. It is very probable that the comparatively low detection with Cy3-monolabeled

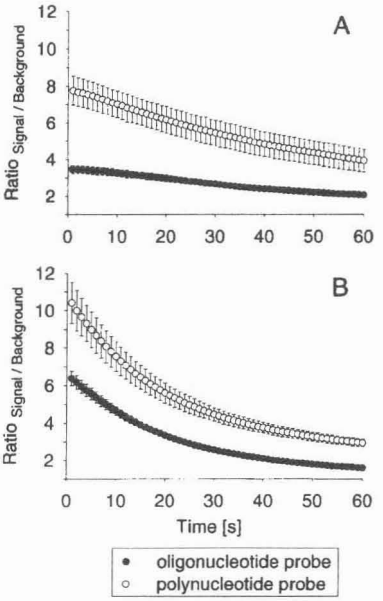


FIG. 3. Bleaching dynamics of North Sea picoplankton enrichments stained with the monolabeled oligonucleotide probe EUB338 or the multilabeled polynucleotide probe EUBAC and labeled with fluorescein (A) or Cy3 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background fluorescence.

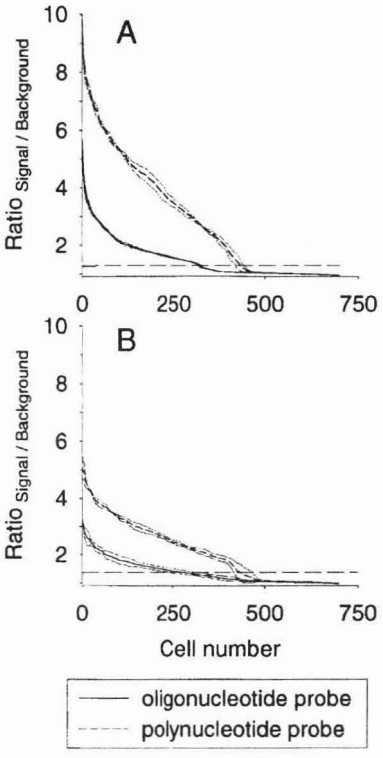


FIG. 4. Signal-to-background ratio spectra of cells hybridized with oligonucleotide probe EUB338 (labeled with Cy3) and polynucleotide probe EUBAC (labeled with fluorescein). Samples were from Monterey Bay from 12 April 2000 (A) or 16 November 2000 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background fluorescence, i.e., cells that were not hybridized. The horizontal broken line indicates the lower limit for positive microscopic counts with probe EUB338. Thin lines are ranges of one standard deviation of triplicate samples.

oligonucleotide probes in German Bight winter samples is in part due to bleaching of the dimmest cell fraction.

At identical camera gain settings and image capture times, the mean fluorescence intensities (mean object grey values) of positively hybridized bacteria in surface samples from 12 April in Monterey Bay (Fig. 4A), stained by oligoFISH or polyFISH, were 72 ± 0.7 and 100 ± 1 (mean ± 1 standard error; n_{EUB} = 1,061, n_{EUBAC} = 1,456). In samples from 16 November (Fig. 4B), the mean fluorescence intensities of probe-positive objects after either staining procedure were statistically indistinguishable (97 ± 0.7 and 100 ± 0.8) (n_{EUB} = 1,314, n_{EUBAC} = 1,248; $P > 0.1$), but oligoFISH with Cy3-labeled oligonucleotides resulted in almost twice the background fluorescence as that after polyFISH. Thus, the mean signal-to-background ratio of EUB338-stained cells both in spring and autumn (2.1 ± 0.03 and 1.7 ± 0.02) was significantly lower than those of EUBAC-stained cells (4.2 ± 0.04 and 2.8 ± 0.02) ($P < 0.01$). The signal-to-background ratio spectrum of oligoFISH-stained cells in general declined more gradually, with little or even no clear difference between the dimmest fraction of probe-positive and probe-negative cells (Fig. 4). This presents a major obstacle for the microscopic evaluation of samples with numerous dimly stained cells and may result in more subjective counting at low EUB338 detection. In contrast, such a threshold could be more readily distinguished after polyFISH staining. As mentioned above, accurate counting of dim oligoFISH-stained cells will also be hampered by a more rapid bleaching within the typical period of time for the microscopic inspection of a single field. Altogether, this implies that the fraction of DAPI-stained cells visualized by oligoFISH in such samples might be strongly influenced both by the quality of the microscopic equipment and by the counting strategy.

Detection of archaea. In North Sea samples, the specific counts with the oligonucleotide probe ARCH915 (archaea) during spring and summer were on average 8% higher than the sum of polynucleotide probes G1 (crenarchaeota) and G2 (euryarchaeota) (Fig. 5). A high percentage of cells hybridizing with ARCH915 in April (26% of DAPI counts) was not detected by polyFISH with G1 (<1%) or G2 (5%). Simultaneous hybridizations of this sample with Cy3-labeled ARCH915 and Cy5-labeled EUB338 and inspection by confocal microscopy revealed a double binding of both probes to some cells (Fig. 6). Considering the systematically lower relative abundances determined with the polynucleotide probes, we suggest that the ARCH915 probe unspecifically binds to some pelagic marine bacteria even under stringent hybridization conditions (41). Therefore, other available (14, 31) or newly designed probes might be more adequate for a future quantification of archaea in marine samples by oligoFISH. Nevertheless, blooms of archaea in North Sea picoplankton were detected by both FISH methods between May and September, reaching a maximum of 31% of DAPI counts as determined with the G2 probe and 41% as determined with ARCH915.

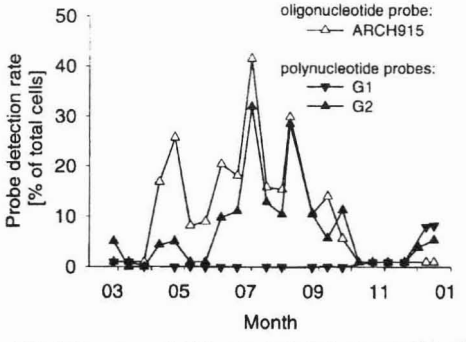


FIG. 5. Percentages of DAPI-stained cells in the German Bight of the North Sea (surface samples, 1998) detected by FISH with oligonucleotide and polynucleotide probes (ARCH915, all archaea; G1, crenarcheota; G2, euryarcheota).

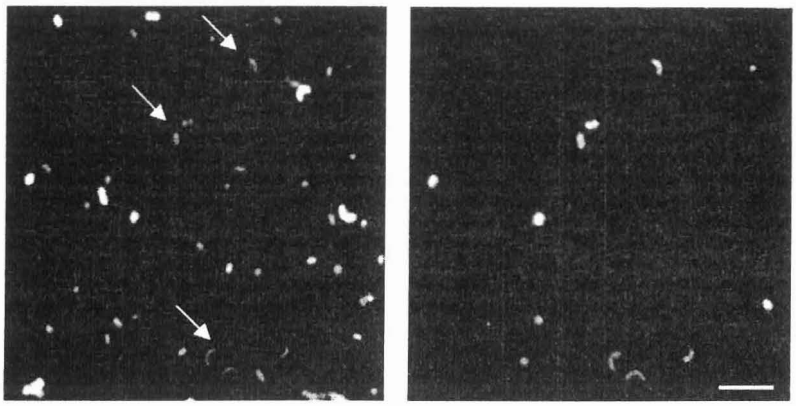


FIG. 6. Confocal laser scanning micrographs of a double hybridization of North Sea picoplankton (July 1998) with oligonucleotide probes EUB338-Cy3 (bacteria, left panel) and ARCH915-Cy5 (archaea, right panel). Arrows indicate cells hybridized by both probes. Bars, 10 μm.

In Monterey Bay samples, the percentage of total counts detected by oligonucleotide probe ARCH915 were high in spring, even exceeding the summed counts of both archaeal polynucleotide probes (Fig. 2). In winter, oligoFISH counts for archaea were below detection limits, whereas archaeal cells stained by polynucleotide probe G1 could still be visualized. Pelagic crenarcheota (cells detected by probe G1) were most abundant in the water column below 100 m depth and represented up to 21 and 33% of DAPI counts in winter and summer, respectively. The G2 probe (euryarcheota) detected 12% of DAPI-stained cells at the surface in summer and less than 1% in winter throughout the sampled water column.

The observed zonation of the two archaeal groups in the depth profiles (Fig. 2A) corresponds well with previous reports about the vertical distribution of pelagic crenarcheota and euryarcheota in the coastal Pacific Ocean and other marine provinces (24–26). In general, G1 archaea (crenarcheota) are regarded as a more prominent component of the marine picoplankton in the deeper water layers (8, 14, 15, 18, 26). In North Sea surface waters, G1 archaea only occurred sporadically, at maximal densities of 3.7×10^3 cells ml^{-1}. In contrast, G2 archaea (euryarchaeota) were detected in high abundances in the shallow and eutrophied coastal North Sea (Fig. 5), reaching total cell numbers between 1×10^5 and 2×10^5 cells ml^{-1}. To our knowledge, this is the first report that a substantial fraction of coastal North Sea picoplankton may be seasonally formed by euryarchaea. PolyFISH results from both the seasonal study and the depth profiles furthermore indicated that the pelagic euryarchaeota were mainly present in the spring or summer plankton but only appeared sporadically during autumn and winter. This may indicate that pelagic archaea are either disproportionately eliminated from the plankton at the end of the productive season or that their productivity decreases significantly below that of the bacteria.

Potential and limitations of either FISH approach. We chose our standard oligoFISH protocol for the comparison of the two approaches (34), using 5′-Cy3-monolabeled probes and direct microscopic evaluation. One advantage of this approach is that the probes can be obtained commercially in high quality at relatively low cost. A modified protocol for oligoFISH in the open ocean reports the use of Cy3 doubly labeled probes in combination with preincubation with chloramphenicol (30) and specialized intensifier equipment for image capture, to visualize "cells with fluorescence considerably below direct detection by eye" (14). However, detection of bacteria and archaea by this modified approach in the San Pedro Channel (approximately 40 to 60% and 15 to 30%, respectively) (14) was not higher than our oligoFISH results in a comparable depth profile from Monterey Bay (Fig. 2A). This indicates that even an oligoFISH staining protocol with greatly enhanced sensitivity would not fundamentally increase the signal-to-noise problem associated with probe sensitivity. High detection of DAPI-stained cells by EUB338 (>70%) using oligoFISH with the original protocol (16) were also reported from surface samples off the California coast (6).

Our study illustrates limitations of oligoFISH with presently available fluorescent dyes and direct microscopic evaluation for the staining of marine picoplankton in deeper water layers and during winter (Fig. 1 and 2). The majority of the microbial communities in such samples, presumably cells with low rRNA content (27), could not be detected. Any analysis of marine picoplankton samples by any of the current oligoFISH approaches should thus be limited to locations and seasons that allow for a detection comparable to that with polyFISH staining. It is, however, difficult to predict the percentages of total counts detectable by oligoFISH in different marine habitats, and there is evidence that high oligoFISH detection of bacterioplankton are not limited to the coastal temperate regions. Simon et al. (39) reported counts with EUB338 in the Antarctic circumpolar vent that exceeded 80% of total picoplankton abundances and increased to >90% at the marginal ice zone.

Our data furthermore indicated that polyFISH is sometimes a superior means to detect and discriminate bacteria and archaea that are low in ribosome content (Fig. 1 and 2). Despite

this obvious advantage, there are also drawbacks to the polyFISH approach. First, the application of oligoFISH probes is not dependent on any other molecular biological technique but mainly relies on the availability of a sequence database and an epifluorescence microscope. In contrast, each batch of polyFISH probes has to be synthesized using PCR and in vitro transcription techniques (8). In addition, RNA polynucleotides are more readily degraded during handling than the DNA oligonucleotide probes, e.g., by repeated freezing and thawing. So far, fluorescently labeled polynucleotides for the detection of marine picoplankton have been designed to distinguish groups that are evolutionarily very distant from each other (8, 18). The probes EUBAC, G1, and G2 are mixes of polynucleotides targeting the entire 16 and 23S rRNAs and, consequently, numerous probe molecules can simultaneously bind to each ribosome. To design more specific polynucleotide probes that discriminate different phylogenetic lineages, highly variable regions of 300 to 600 nucleotides within the 16S or 23S rRNA should be targeted (M. Leclerc, personal communication) (23, 40, 42). Nevertheless, oligoFISH may still offer higher phylogenetic resolution than polyFISH, and conditions for specific hybridization must be rigorously tested. In some cases it has been shown that the hybridization conditions required for a clear discrimination between closely related groups may substantially decrease polyFISH probe fluorescence intensity (42). In summary, any future improvement of the FISH approach for marine microbiology should attempt to preserve the superior signal intensity of multilabeled polynucleotides and at the same time reach the specificity of the oligonucleotide probes.

ACKNOWLEDGMENTS

We thank K.W. Klings (Biologische Anstalt Helgoland) for help during sampling and Marion Leclerc for help with probe synthesis. The officers and crew of the RV *Point Lobos* are acknowledged for sampling opportunities at sea.

This study was supported by the German Ministry of Education and Science (BMBF 01 LC0021/TP4) and the Max Planck Society.

REFERENCES

1. **Amann, R., F. O. Glöckner, and A. Neef.** 1997. Modern methods in subsurface microbiology—in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol. Rev. **20:**191–200.
2. **Amann, R. I.** 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Mol. Microb. Ecol. Manual **3.3.6:**1–15.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
5. **Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jorgensen, U. Witte, and O. Pfannkuche.** 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407:**623–626.
6. **Cottrell, M. T., and D. L. Kirchman.** 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. **66:**5116–5122.
7. **Cottrell, M. T., and D. L. Kirchman.** 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. **66:**1692–1697.
8. **DeLong, E. F., L. T. Taylor, T. L. Marsh, and C. M. Preston.** 1999. Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Appl. Environ. Microbiol. **65:**5554–5563.
9. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
10. **Eilers, H., J. Pernthaler, and R. Amann.** 2000. Succession of pelagic marine bacteria during enrichment: a close look on cultivation-induced shifts. Appl. Environ. Microbiol. **66:**4634–4640.
11. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
12. **Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann.** 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. **67:**5134–5142.
13. **Faude, U., and M. G. Höfle.** 1997. Development and application of monclonal antibodies for in situ detection of indigenous bacterial strains in aquatic ecosystems. Appl. Environ. Microbiol. **63:**4534–4542.
14. **Fuhrman, J. A., and C. C. Ouverney.** 1998. Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native Archaea with fluorescent single cell probes. Aquat. Ecol. **32:** 3–15.
15. **Fuhrman, J. A., and A. A. Davis.** 1997. Widespread Archaea and novel bacteria from the deep sea as shown by 16S rRNA gene sequences. Mar. Ecol. Prog. Ser. **150:**275–285.
16. **Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K.-H. Schleifer.** 1996. An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst. Appl. Microbiol. **19:**403–406.
17. **Glöckner, F. O., B. M. Fuchs, and R. Amann.** 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. **65:**3721–3726.
18. **Karner, M., E. F. DeLong, and D. M. Karl.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409:**507–509.
19. **Karner, M., and J. A. Fuhrman.** 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl. Environ. Microbiol. **63:**1208–1213.
20. **Lanoil, B. D., and S. J. Giovannoni.** 1997. Identification of bacterial cells by chromosomal painting. Appl. Environ. Microbiol. **63:**1118–1123.
21. **Lee, N., P. Nielsen, K. Andreasen, S. Juretschko, J. Nielsen, K. Schleifer, and M. Wagner.** 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. **65:**1289–1297.
22. **Llobet-Brossa, E., R. Rosselló-Mora, and R. Amann.** 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. **64:**2691–2696.
23. **Ludwig, W., S. Dorn, N. Springer, G. Kirchhof, and K. H. Schleifer.** 1994. PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl. Environ. Microbiol. **60:**3234–3244.
24. **Massana, R., E. F. DeLong, and C. Pedros-Alio.** 2001. A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. Appl. Environ. Microbiol. **66:**1777–1787.
25. **Massana, R., A. E. Murray, C. M. Preston, and E. F. DeLong.** 1997. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara channel. Appl. Environ. Microbiol. **63:**50–56.
26. **Murray, A. E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge, and E. F. DeLong.** 1999. A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. Aquat. Microb. Ecol. **20:**129–145.
27. **Oda, Y., S. J. Slagman, W. G. Meijer, L. J. Forney, and J. C. Gottschal.** 2000. Influence of growth rate and starvation on fluorescent in situ hybridization of Rhodopseudomonas palustris. FEMS Microbiol. Ecol. **32:**205–213.
28. **Oerther, D. B., J. Pernthaler, A. Schramm, R. Amann, and L. Raskin.** 2000. Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Appl. Environ. Microbiol. **66:**2154–2165.
29. **Ouverney, C. C., and J. A. Fuhrman.** 1999. Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. **65:**1746–1752.
30. **Ouverney, C. C., and J. A. Fuhrman.** 1997. Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Appl. Environ. Microbiol. **63:**2735–2740.
31. **Ouverney, C. C., and J. A. Fuhrman.** 2000. Marine planktonic *Archaea* take up amino acids. Appl. Environ. Microbiol. **66:**4829–4833.
32. **Pernthaler, A., J. Pernthaler, H. Eilers, and R. Amann.** 2001. Growth patterns of two marine isolates: adaptations to substrate patchiness? Appl. Environ. Microbiol. **67:**4077–4083.
33. **Pernthaler, J., A. Alfreider, T. Posch, S. Andreatta, and R. Psenner.** 1997. In situ classification and image cytometry of pelagic bacteria from a high mountain lake (Gossenköllesee, Austria). Appl. Environ. Microbiol. **63:**4778–4783.
34. **Pernthaler, J., F. O. Glöckner, W. Schönhuber, and R. Amann.** 2001. Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, p. 207–226. *In* J. H. Paul (ed.), Methods in microbiology, vol. 30. Academic Press, San Diego, Calif.
35. **Pernthaler, J., F. O. Glöckner, S. Unterholzner, A. Alfreider, R. Psenner,**

and R. Amann. 1998. Seasonal community and population dynamics of pelagic *Bacteria* and *Archaea* in a high mountain lake. Appl. Environ. Microbiol. **64:**4299–4306.

36. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
37. **Ravenschlag, K., K. Sahm, and R. Amann.** 2001. Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). Appl. Environ. Microbiol. **67:**387–395.
38. **Rosselló-Mora, R., B. Thamdrup, H. Schäfer, R. Weller, and R. Amann.** 1999. The response of the microbial community of marine sediments to organic input under anaerobic conditions. Syst. Appl. Microbiol. **22:**237–248.
39. **Simon, M., F. O. Glöckner, and R. Amann.** 1999. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. Aquat. Microb. Ecol. **18:**275–284.
40. **Spring, S., U. Lins, R. Amann, K. H. Schleifer, L. C. S. Ferreira, D. M. S. Esquivel, and M. Farina.** 1998. Phylogenetic affiliation and ultrastructure of uncultured magnetic bacteria with unusually large magnetosomes. Arch. Microbiol. **169:**136–147.
41. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley & Sons Ltd., Chichester, England.
42. **Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger, and K.-H. Schleifer.** 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. **60:**3228–3235.
43. **Weinbauer, M. G., and M. G. Höfle.** 1998. Distribution and life strategies of two bacterial populations in a eutrophic lake. Appl. Environ. Microbiol. **64:**3776–3783.

3

Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria

Annelie Pernthaler, Jakob Pernthaler, Rudolf Amann.

Applied & Environmental Microbiology. In press.

Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria

Annelie Pernthaler, Jakob Pernthaler, and Rudolf Amann

Molecular Ecology Group, Max-Planck-Institute for Marine Microbiology, 28359 Bremen, Germany

Fluorescence in situ hybridization (FISH) with horseradish peroxidase (HRP) labeled oligonucleotide probes and tyramide signal amplification (TSA), also known as CARD, is presently not generally applicable to heterotrophic bacteria in marine samples. Penetration of the HRP molecule into bacterial cells requires permeabilization procedures that cause high and most probably species-selective cell loss. Here we present an improved protocol for CARD-FISH of marine planktonic and benthic microbial assemblages. After concentration of samples onto membrane filters and subsequent embedding of filters in low gelling point agarose no decrease in bacterial cell numbers was observed during 90 minutes of lysozyme incubation (10 mg ml^{-1}, 37°C). The detection rates of coastal North Sea bacterioplankton by CARD-FISH with a general bacterial probe (EUB338-HRP) were significantly higher (mean, 94% of total cell counts; range, 85 - 100%) than with a monolabeled probe (EUB338-mono; 48%, 19 - 66%). Virtually no unspecific staining was observed after CARD-FISH with an antisense EUB338-HRP. Members of the marine SAR86 clade were undetectable by FISH with a monolabeled probe, yet a substantial population was visualized by CARD-FISH (7%, 3 – 13%). Detection rates of EUB338-HRP in Wadden Sea sediments (81%, 53 - 100%) were almost twice as high as of EUB338-mono (44%, 25 - 71%). The enhanced fluorescence intensities and signal to background ratios makes CARD-FISH superior to FISH with directly labeled oligonucleotides for the staining of bacteria with low rRNA content in the marine environment.

Fluorescence in situ hybridization (FISH) of bacteria was first described more than a decade ago (2, 11), and was hailed as a breakthrough for microbial ecology. However, researchers initially encountered difficulties when applying the method to environmental samples other than from highly eutrophic systems. The majority of bacteria in aquatic habitats is small, slowly growing or starving (31), and the signal intensities of hybridized bacterioplankton cells were frequently below detection limits or lost in high background fluorescence. During the last years numerous efforts have been made to increase the sensitivity of FISH: brighter fluorochromes (1, 19), image intensified video microscopy (17), chloramphenicol treatment to increase the rRNA content of the growing bacterial fraction (35), hybridization with more than one fluorescently labeled oligonucleotide probe (27, 29), helper oligonucleotide probes (15, 21), multiply-labeled polyribonucleotide probes (10, 25, 36), and signal amplification using reporter enzymes (27, 40).

The tyramide signal amplification (TSA), also known as catalyzed reporter deposition (CARD) has been introduced more than a decade ago (5) for immunoblotting and immunosorbent assays, using horseradish peroxidase (HRP) and haptenized tyramines. CARD is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity. Tyramines are phenolic compounds and HRP can catalyze dimerization of such compounds when they are present in high concentrations, probably by the generation of free radicals (49). If applied at lower concentrations, such as in the signal amplification reaction, the probability of dimerization is reduced, whereas the binding of the highly reactive intermediates to electron-rich moieties of proteins, such as tyrosine, at or near the site of the peroxidase binding site is favoured. In this way, if fluorochrome-labeled tyramides are used, numerous fluorescent molecules can be introduced at the hybridization site in situ. This results in greatly enhanced FISH sensitivity as compared to probes with a single fluorochrome. CARD in combination with nucleotide probes and/or antibodies is routinely used in histology and cytochemistry to localize specific nucleic acid sequences (DNA, RNA) in microscopic preparations of tissues, cells, and chromosomes, and allows the detection of rare and even single-copy-number targets (mRNAs, genes) (6). To date, a wide variety of research and diagnostic applications have been described, making this technique an integral part of studies of gene mapping, gene expression, RNA processing and transport, the three-dimensional organization of the nucleus, tumor genetics, microbial infections, and prenatal diagnosis (43).

Unfortunately, this signal amplification technique is presently not applicable to heterotrophic bacteria in environmental samples (40). The critical step of this approach is

the diffusion of large molecules such as enzymes, antibodies, or (strept)avidin into whole fixed cells (4). It is usually necessary to include a very carefully controlled permeabilization step prior to enzymatic signal amplification, balancing permeability with cellular integrity (41). Since cell wall composition varies greatly among procaryotes, such procedures usually compromise the universal applicability of this approach in mixed microbial communities (40).

In this study, we modified preparation and permeabilization procedures as well as staining protocols for FISH with HRP labeled oligonucleotide probes and CARD for the quantification of planktonic and benthic marine bacteria. This lead to a significant improvement of detection rates, as compared to our current protocol for FISH with monolabeled probes (37).

MATERIALS AND METHODS

Sample collection and preparation. Bacterioplankton samples originated from a cruise on the RV Uthörn between October 1 and 3, 1999. On 3 consecutive days samples were collected from horizontal transects between the island of Helgoland and the estuaries of the rivers Elbe and Eider, respectively (days 1,2), and from Helgoland into open North Sea waters (day 3) (Fig. 1). Samples were fixed for 1 hour in particle-free formaldehyde solution (final concentration, 2% volume/volume, v/v) and were prefiltered through cellulose nitrate membrane filters (pore size, 3 μm, Sartorius, Göttingen, Germany). For FISH, portions of 10 ml were filtered onto white polycarbonate membrane filters (type GTTP, pore size, 0.2 μm, size, 47 mm, Millipore, Eschborn, Germany), washed with 5 ml of distilled water, and stored at –20°C until further processing. Additional subsamples (2 ml) were filled in reaction vials and stored at –20°C for flow cytometric determination of total cell counts.

Sediment samples were collected on February 20, 2001 from a near shore intertidal mud flat at Dangast, located in the Jadebusen Bay of the German Wadden Sea. Sediment cores were sliced in 0.5 cm sections and fixed in 4% (v/v) formaldehyde solution. Subsamples were diluted, sonicated and filtered onto white membrane filters (type GTTP, pore size, 0.2 μm, size, 25 mm, Millipore) as described (37).

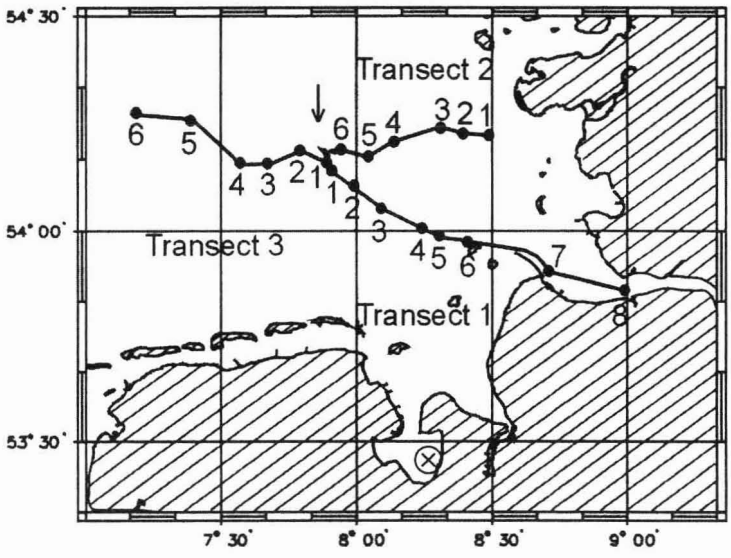


Figure 1. Map of the German Bight of the North Sea indicating the sampling locations along the three transects (numbers) and the site of sediment sampling (⊗). Arrow points at the location of the island of Helgoland.

Total cell counts. Total picoplankton cell numbers in the <3 μm water fraction was determined flow cytometrically as decribed (12). For quantification of potential cell loss during lysozyme treatment (see below), plankton samples were filtered onto polycarbonate filters (type GTTP, diameter, 25 mm, pore size, 0.2 μm, Millipore), using gentle vacuum and cellulose nitrate support filters (pore size, 0.45 μm, Sartorius) to optimize the distribution of cells on filters. Filters were subsequently washed twice with 5 ml ultrapure water (MQ, Millipore). Next the filters were either embedded in agarose (see below) or left unembedded, treated with lysozyme for 0, 40, and 90 minutes, and hybridized with the HRP-labeled probe EUB338 (2) as described below. All preparations were done in triplicates.

FISH with Cy3 labeled oligonucleotide probes. Sections of filters were hybridized with probes EUB338, NON338 (3), ROS537 (14), SAR86-1249 (13) as described previously (19). Oligonucleotides labeled with the cyanine dye Cy3 were purchased from ThermoHybaid (Interactiva Division, Ulm, Germany).

Sample prozessing for hybridization with HRP-labeled probes. A detailed protocol of all steps of sample processing for CARD-FISH is given in Table 1. To avoid cell loss during cell wall permeabilization, filters were dipped in low gelling point agarose

(0.2% [weight/volume, w/v] in MQ, MethaPhor, Bioproducts, Rockland, Maine), dried face up on glass slides at 35°C and subsequently dehydrated in 96% [v/v] ethanol for 1 min. To inhibit endogenous peroxidases, samples from Wadden Sea sediment were treated over night with 0.1% active diethylpyrocarbonate (DEPC, [w/v], Fluka, Taufkirchen, Germany) in phosphate buffered saline (PBS, 145 mM NaCl, 1.4 mM NaH2PO4, 8 mM Na2HPO4, pH 7.4) at 37°C. For cell wall permeabilization, filters were incubated in a lysozyme solution (Fluka, 10 mg ml-1 in 0.05 M EDTA, 0.1 M TrisHCl [pH 7.5]) at 37°C for at least 30 min. The sections were subsequently washed with MQ, dehydrated with 96% ethanol, dried at room temperature and subsequently stored in petri dishes at –20°C until further processing.

FISH with HRP-labeled oligonucleotide probes. For FISH with the probes EUB338, NON338, ROS537, SAR86-1249 labeled with HRP, 10 to 20 filter sections were placed in a 0.5 ml reaction vial. 400 μl of hybridization buffer (0.9 M NaCl, 20 mM TrisHCl [pH 7.5], 10% dextrane sulfate [w/v], 0.02% [w/v] sodium dodecyl sulfate [SDS], 55% [v/v] formamide [Fluka], 1% [w/v] Blocking Reagent [Boehringer, Mannheim, Germany], 0.5 mg ml-1 Salmon Sperm DNA [Boehringer], 0.5 mg/ml Escherichia coli tRNA [Boehringer]) and 4 μl of HRP-probe working solution (50 ng μl-1, ThermoHybaid) were pipetted onto the filter sections. The reaction vial was incubated at 35°C for 2 hours, then the filter sections were removed from the hybridization mixture, and incubated in 50 ml of prewarmed washing buffer (3 mM NaCl, 5 mM EDTA [pH 8.0], 20 mM TrisHCl [pH 7.5], 0.01% [w/v] SDS) at 35°C for 10 min. Formamide concentration of 55% was used for all probes. This allowed a specific discrimination of one mismatch, as tested by hybridizations of pure cultures of Pseudoalteromonas sp. with the probes Bet42a and Gam42a (data not shown). The hybridization buffer was prepared as follows: MQ, dextrane sulfate, NaCl, SDS and TrisHCl were mixed, brought in solution at 60°C, and subsequently cooled down on ice. Formamide, Blocking Reagent, Salmon Sperm DNA, and Escherichia coli tRNA were than added. The hybridization buffer was then stored at –20°C for up to 3 months.

Table 1. Summary of steps for FISH and CARD of marine bacteria

Embedding	1. prepare subsamples on membrane filters (ref. 37) 2. dip filters in 0.2% low gelling point agarose, place filters face up onto glass slides and air dry at 35°C 3. dehydrate in 96% ethanol (1 min, room temperature [RT]) 4. air dry [1)]
Permeabilization and inactivation of peroxidases	5. incubate in lysozyme (37°C, > 30 min) 6. sediment samples: incubate in 0.1% active DEPC in PBS (37°C, over night) 7. wash twice in MQ (1min, RT) 8. wash in 96% ethanol (1min, RT) 9. air dry filters [1)]
Hybridization	10. cut filters in sections 11. place sections in reaction vial (0.5 ml, 10 – 20 sections per vial) 12. mix 400 μl of hybridization buffer and 4 μl of probe working solution and add to filter sections 13. incubate at 35°C for at least 2 hours 14. wash filters in prewarmed washing buffer (10 min, 35°C), do not air dry filter sections after washing
Tyramide signal amplification	15. remove excess liquid with blotting paper, but don`t let filters run dry 16. incubate in 1 x PBS amended with 0.05% of Triton X-100 (10 ml, RT, 15 min, mild agitation) 17. dab filters on blotting paper, but don`t let run dry 18. incubate in substrate mix (1 part Cy3-tyramide, 10 parts of amplification diluent) (RT, 10 min, in the dark) 19. dab filter on blotting paper 20. wash in 10 ml 1 x SSC, (RT, 10 min, in the dark, mild agitation) 21. wash in 10 ml MQ (RT, 1 min) 22. wash in 10 ml 96% ethanol (RT, 1 min) 23. air dry preparations [1)] 24. counterstain with DAPI [1)]

[1)] preparations may be stored at –20°C for several days to weeks without apparent loss in signal

In situ detection of HRP-conjugated oligonucleotide probes. To equilibrate the probe-delivered HRP, sections were placed in 10 ml of 1x PBS amended with 0.05% of Triton X-100 for 15 min at room temperature. To remove excess buffer, the filter sections were dabbed onto blotting paper, and immediately transferred to a substrate mix containing 1 part of tyramide-Cy3 and 10 parts of amplification buffer (TSAdirect, NEN Lifescience Products, Boston, Maine USA) and incubated for 10 min at room temperature in the dark. Filter sections were then briefly placed on blotting paper to remove excess tyramide-Cy3 and washed at room temperature in the dark in 10 ml of 1x PBS amended with 0.05% of Triton X-100, MQ, and 96% ethanol, respectively. To decrease background fluorescence, sections were subsequently washed in MQ and 96% ethanol for 1 min, respectively. Afterwards, filter sections were air dried and stored at – 20°C until further processing (within 2 days).

In order to test if higher substrate concentration would increase detectability, a concentration series of tyramide-Cy3 was performed using the following dilutions: 1+200, 1+100, 1+50, 1+20, 1+10 (parts of tyramide-Cy3 + parts of amplification buffer, respectively).

Microscopic evaluation. Filter sections were covered in mountant (5.5 parts of Citifluor [Citifluor Ltd., London, U.K.], 1 part of VectaShield [Vector Laboratories, Burlingame, CA], 0.5 parts of 1 x PBS, amended with 4´,6´-diamidino-2-phenylindol [DAPI, final concentration 1 μg / μl]) and evaluated on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany), equipped with a HBO 100 W Hg vapor lamp, appropiate filter sets for Cy3 and DAPI fluorescence (20), and a 100x Plan Apochromat objective. Between 600 and 800 DAPI stained objects were counted per sample.

Statistical evaluation. Statistical evaluation was carried out with the software STATISTICA (ver 5.0, StatSoft, Tulsa, OK). We tested if the percentage of DAPI counts detected by probe EUB338 and CARD-FISH were different from those with monolabeled EUB338 in plankton and sediment samples. The Wilcoxon matched pair test was used as nonparametric alternative to the Student's t-test for dependent samples.

RESULTS

Optimization of sample pretreatment, hybridization, and substrate reaction for CARD-FISH. Initial experiments for method developement were carried out with a HRP labeled oligonucleotide probe targeting *Bacteria* (EUB338) and surface bacterioplankton samples from the North Sea. To optimize the permeabilization of bacterial cell walls, different chemicals and enzymes were examined, such as SDS, formamide Triton X-100, acetone, proteinase K and lysozyme. The latter showed the best results with respect to detection rate and was therefore used for CARD-FISH. In order to quantify potential cell loss, a lysozyme incubation time series was performed. We could not detect significant cell loss (DAPI total counts) in agarose embedded samples even after 90 min of lysozyme treatment (Fig. 2). In contrast, a strong decline in cell numbers was observed in samples that were not embedded, and after 90 minutes of lysozyme incubation $^2/_3$ of cells were lost from the filters. Samples that were not treated with lysozyme showed lower detection rates and hybridization signals after CARD-FISH. Detection rates of a EUB338-HRP with CARD increased from 46% (± 2) of DAPI stained cells in the unpermeabilized samples to 86% (± 5) after 40 min of permeabilization (Fig. 2).

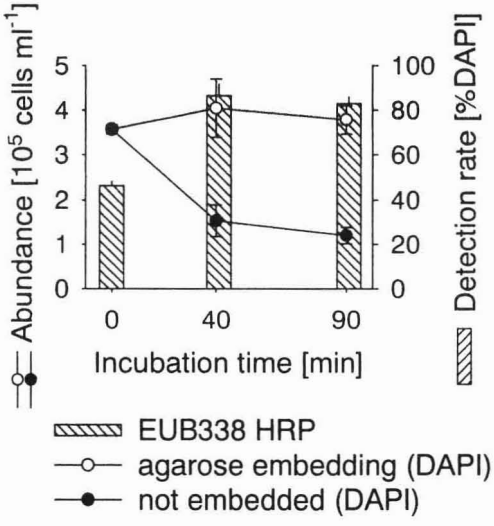


Figure 2. Lines & symbols: Abundances of bacterioplankton cells on membrane filters during incubation with lysozyme (10 mg ml^{-1}, 37°C) with and without enbedding in low-gelling-point agarose. Bars: %FISH detection rates with a HRP labeled probe and CARD in embedded samples. Errorbars indicate either standard deviations (abundances) or total ranges (percentages) of triplicates.

At probe concentration (2 – 5 ng/μl), recommended by many FISH protocols (19) we observed numerous unspecific fluorescent deposits after CARD-FISH. In order to decrease background, we lowered the probe concentration ten-fold (48). This resulted in elimination of non-specific deposits of Cy3-tyramide without decreasing signal intensities or detection rates (data not shown). We also varied the tyramide concentration in the substrate mix for TSA (Fig. 3). At tyramide dilutions recommended by the manufacturer (1:50-1:200) we could only detect 37% to 66% of DAPI stained cells (Fig. 3). After increasing the tyramide concentration to 1:11 and 1:21 the abundances of probe positive cells increased to 86% and 71% DAPI, respectively, without substantial increase of background fluorescence. Higher tyramide concentrations than 1:11 resulted in a high background flourescence (data not shown). A prolonged hybridization period also positively influenced the detection rates (Table 2), yet longer hybridization periods than 2 h did not further raise detection rates (data not shown). For subsequent quantification of North Sea bacterioplankton and Wadden Sea bacteriobenthos by CARD-FISH we used the optimal set of conditions as determined in our preceeding experiments (see also Table 1).

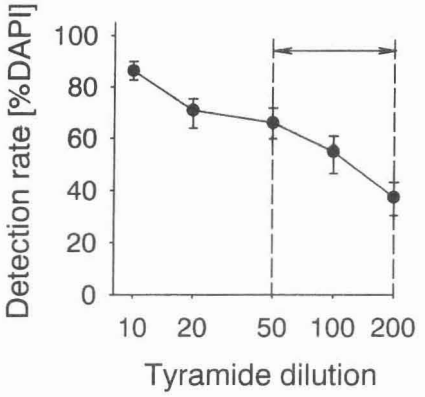


Figure 3. FISH detection rates with HRP-labeled probes and CARD at increasing dilution of tyramide substrate in plankton samples. Numbers on x-axis indicate parts of amplification diluent added to 1 part of tyramide-Cy3. Arrow: range of tyramide concentrations recommended by the manufacturer. Errorbars: ranges of triplicates.

Quantification of cells in environmental samples. The densities of heterotrophic picoplankton in 3 μm prefiltered water along the 3 transects ranged between 1.7 and 9.9 x 10^5 cells ml-1, and abundances were highest in samples that were closest to the coast (Table 3). In all these samples bacterial cells detectable by FISH with EUB338-HRP

accounted for the majority (mean 94%; range: 85 - 100%) of DAPI stained cells (Fig. 4). The detection rates with monolabeled EUB338-Cy3 were significantly lower (48%, 19 - 66%) ($n = 20$, $p<0.001$) (Fig. 4). A highly significant difference in detection rates was also observed in Wadden Sea sediment samples from February 2001 ($n = 10$, $p < 0.01$) (Fig. 5). In the permanently mixed upper sediment layers (0 – 6 cm) cell densities determined with EUB338-HRP (93%, 87 - 100%) were almost twice as high as by EUB338-Cy3 (51%, 35 - 72%). In the deeper layers the detection rates with both, EUB338-HRP (63%, 53 - 74%) and EUB338-Cy3 (31%, 26 - 39%) were lower than in the upper layers.

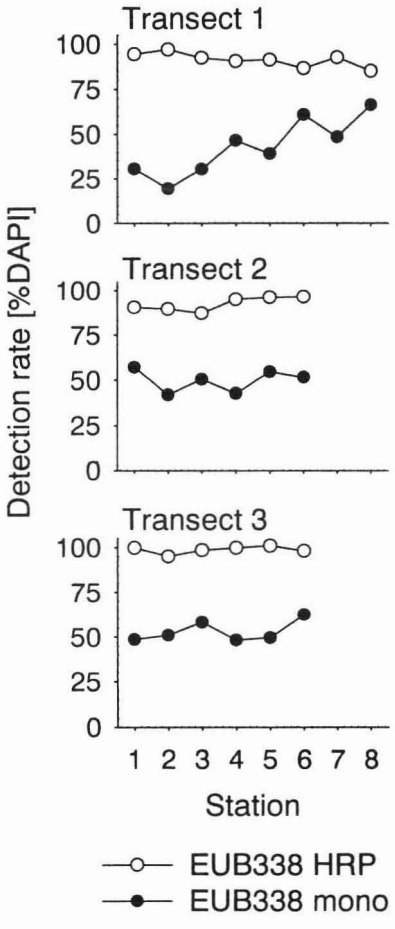


Figure 4. Comparison of detection rates by FISH with a Cy3-monolabeled general bacterial probe (EUB338) and CARD FISH with a HRP labeled probe along 3 transects in North Sea surface water samples from Oktober 1999.

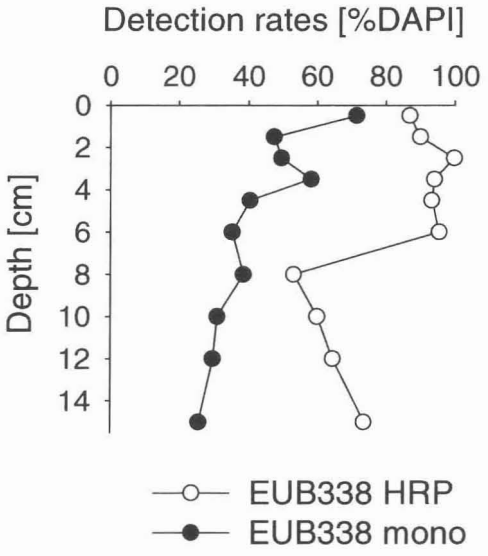


Figure 5. Comparison of detection rates by FISH with a Cy3-monolabeled general bacterial probe (EUB338) and CARD FISH with a HRP labeled probe in Wadden Sea sediment samples from February 2001.

In samples from both the water column and the sediment the fluorescence intensities of single cells and the signal to background ratios were much greater with HRP labeled probes than with monolabeled probes (Fig 6). Consequently, the discrimination between probe-positive and probe-negative cells was much easier with HRP labeled probes, and therefore quantification was much faster than with singly labeled probes.

For the determination of unspecific binding of HRP labeled probes and unspecific substrate precipitation by endogenous peroxidases and/or pseudoperoxidase activities all samples were also hybridized with a antisense EUB338, NON338-HRP. We found no probe-positive cells in the transect samples (20 samples, 10 microscopic fields inspected per sample) and altogether 4 probe-positive cells in all sediment samples (10 samples, 10 microscopic fields inspected per sample).

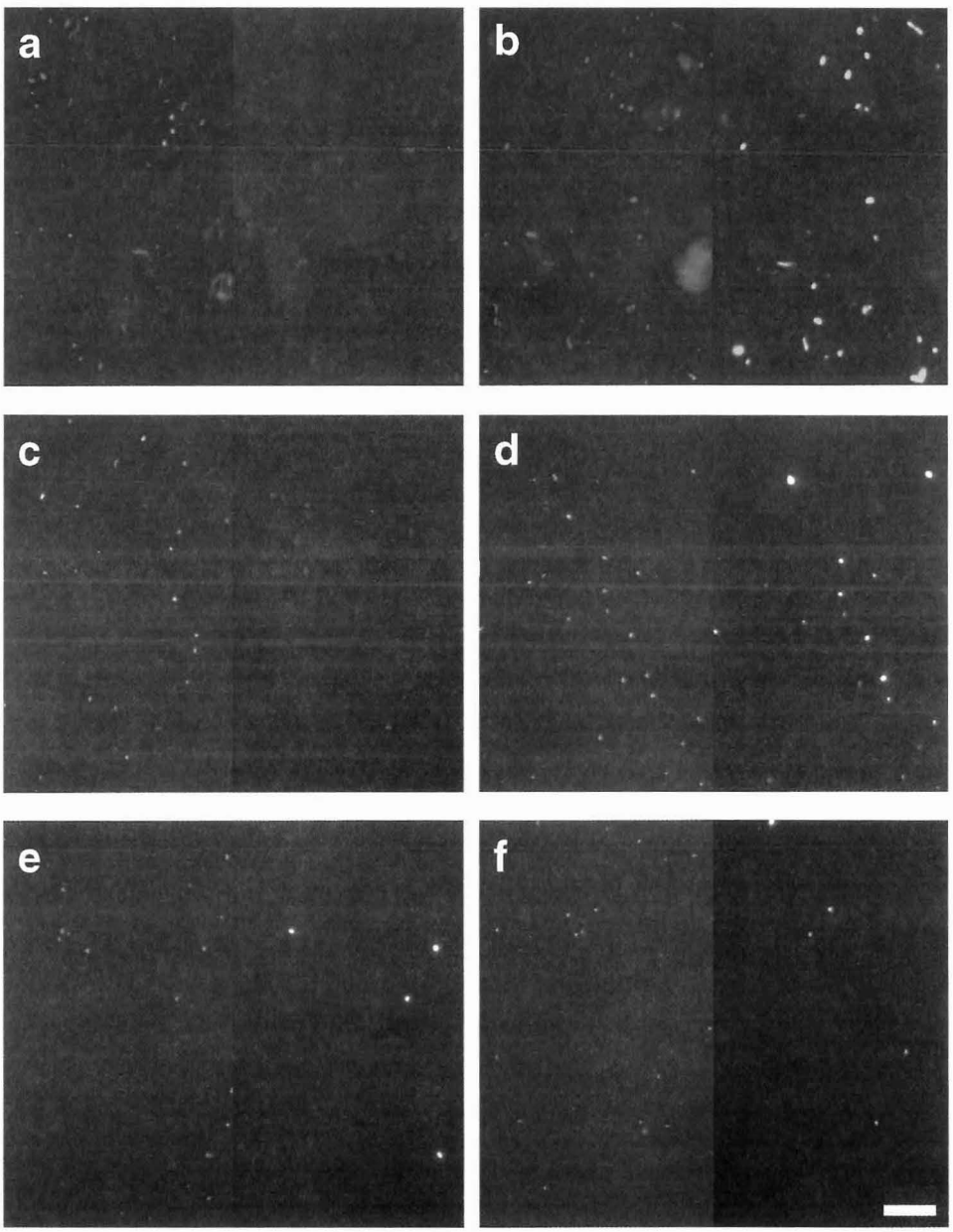
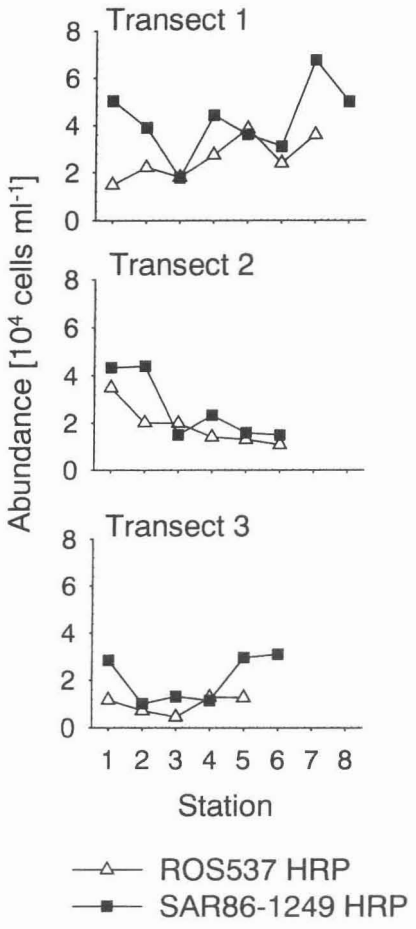


Figure 6: Photomicrographs of FISH stained marine bacteria. Each double panel depicts DAPI staining in blue (left) and probe staining in red (right). Exposure times for images of FISH staining with Cy-3 monolabeled probes (FISH-mono) were 10× those for CARD-FISH staining. Panels (a) to (d): FISH with the general bacterial probe EUB338; (a) sediment, FISH-mono; (b) sediment, CARD-FISH; (c) plankton, FISH-mono; (d) plankton, CARD-FISH; (e) plankton, CARD-FISH with ROS537, specific for members of the *Roseobacter* lineage; (f) plankton, CARD-FISH with SAR86-1249, specific for members of the SAR86 clade. Scale bar: 10 μm



In samples from the transect the detection rates of members of the group *Roseobacter* with either CARD-FISH or a Cy3-monolabeled probe were statistically undistinguishable (n = 20, p = 0.84) (Table 2), and both techniques stained a morphologically homogenous cell population of 0.5 - 3.9 x 10^5 cells ml^{-1} (Figs. 6, 7). The highest *Roseobacter* abundances were found nearest to the coast, and this group closely followed the patterns of total cell numbers (Spearman rank correlation, n = 20, r = 0.89, p< 0.001) (Fig. 7, Table 2). Using a Cy3 -monolabeled probe specific for members of the SAR86 clade (13) we could not detect any stained cells. In contrast, relative abundances of up to 13% of DAPI counts could be observed after CARD-FISH with a HRP labeled probe, and SAR86 exhibited pronounced fluctuation in cell numbers across the three transects (Fig. 7, Table 2). Cells detected with probe SAR86-1249-HRP were morphologically uniform (Fig. 6)

Figure 7. Abundances of members of the SAR86 (probe SAR86-1249) and *Roseobacter* clades (probe ROS537), with specific HRP-labeled probes and CARD-FISH along 3 transects in surface water samples from the North Sea, taken in Oktober 1999.

We also tested our protocoll on samples from Monterey Bay, California (depth profile, 0 – 200 m). Below 50 m depth the densities of positive cells after CARD-FISH were comparable to those from hybridizations with a multiply labeled polyribonucleotide probe targeted to *Bacteria* (10, 36), whereas only few cells were detectable with Cy3 monolabeled probes (data not shown).

DISCUSSION

In this study, we modified and extended existing protocols using HRP labeled oligonucleotides and CARD for the identification of single procaryotic cells. The combination of several small but significant modifications (Table 1) allowed a substantial increase of FISH sensitivity in marine bacterioplankton and – benthos, as compared to previous reports (27, 40). The attachment of cells onto polycarbonate filters with freshly prepared low-gelling-point agarose was found to be crucial to prevent cell loss during a permeabilization step that was required for high FISH detection rates (Fig. 2). Lowering the probe concentration 10-fold helped to eliminat high background fluorescence without loss of signal intensity of the specifically stained cells, and increasing the tyramide concentration also resulted in significantly increased detection (Fig. 3). Negative controls without probe or with probe NON338-HRP consistently yielded very few or no fluorescently labeled cells.

Using our CARD-FISH protocol we were able to detect the majority of DAPI stained cells in plankton samples, suggesting that the permeabilization procedure is sufficient for most bacteria in North Sea surface waters and for surface Wadden Sea sediments. However, the protocoll was not specifically tested for either archaea, planctomyces or Gram positive bacteria, which may be present in the marine environment (9, 13, 16, 25, 38). We suggest that permeabilization has to be substantially modified for the detection of these groups (47). Nevertheless, our results are consistent with previously reported low abundances of marine archaea in coastal surface waters during autumn (33, 36), and presently there is no evidence that *Actinobacteria* occur in high densities in the marine plankton. The comparatively low CARD-FISH detection rates in the permanently stratified, deeper layers of Wadden Sea sediments (Fig. 5) might be due to the presence of inadaequately permeabilized *Archaea* or *Actinobacteria*, or due to other groups that are not targeted by the bacterial probe EUB338, such as *Planctomycetales* or *Veruccomicrobiales* (8). Alternatively, higher relative abundances of dead or empty cells (23) might also be present in deeper sediment layers.

It has been shown in pure cultures that FISH reliably detects subpopulations with higher rRNA contents, but may miss non growing or starving cells (12, 34). Since only a fraction of bacteria in the marine pelagic environment is growing (18, 26, 28) FISH counts with monolabeled probes may not show the true abundances of particular target organisms in oligotrophic environments (36). In fact, whole populations of particular taxa, such as members of the ubiquitous marine SAR86 clade (32), may remain completely undetected

by FISH with directly Cy3-labeled probes (Table 2). SAR86 has been show to be abundant in different marine surface waters (7, 22, 44). Members of this lineage could be detected in coastal North Sea surface plankton during summer, when FISH detection rates with monolabeled probes exceeded 70%, but not in spring, autumn or winter samples (13). In contrast, a substantial population was readily visualized and quantified by CARD-FISH even in October (Figs. 6,7). Microbes belonging to SAR86 are apparantly not reliably detected with monolabled fluorescent probes. This conclusion is also supported by the complete absence of SAR86 in only some of the stations along a coastal transect in the Pacific Ocean (7).

The application of single monolabeled oligonucleotide probes for the direct microscopic visualization of bacteria in the marine environment is, therefore, probably limited to active, relatively rRNA-rich cells in surface waters, and to particular productive regions, seasons or sediments (30, 36, 42). In addition, it is sometimes hardly predictable if bacterial populations are only partially or if they are quantitatively detected by this FISH approach. For example, members of the *Roseobacter* lineage coud be adaequately quantified both by singly labeled probes and by CARD-FISH (Fig. 7, Table 2) even in plankton samples in which the general FISH detection rates were <50% of DAPI-stained objects (Fig. 4).

Recently, multi-labeled polyribonucleotide probes (10, 25, 36) have been introduced to overcome this limitation, and other techniques for the quantification of population sizes, such as the 5' nuclease assay (45), are also unaffected by low bacterial activity. In many instances our protocol might provide a technically less demanding alternative to the above approaches. CARD-FISH could furthermore be attractive to researchers because an increasing number of well-tested oligonucleotide probes are available for different marine environments (13, 39, 50) and the design of new probes is relatively simple (37). In addition, there are well-established protocols for the conjugation of tyramine with fluorescent dyes (24), and in our laboratory we were able to custom label tyramides with Cy3 and various ALEXA dyes at a fraction of the costs of the commercial products.

The potential of the CARD approach is not limited only to the staining of rRNAs. If whole fixed cells are made accessible for enzymes and/or antibodies, a wide range of methods, routinely used in histology and cytology could also be applied in environmental microbiology, such as detection of mRNAs (47). Many mRNA species are present in abundances ranging from 1 to 1000 copies per cell and therefore are not detectable for most non-radioactive in situ assays. Various CARD-FISH methods for the detection of

mRNA in single cells might be developed, depending on the sample and the target-copy-number. For example, it is not only feasible to hybridize with oligonucleotide probes directly labeled with HRP, but also with digoxigenin (DIG) labeled polynucleotide probes followed by an antibody reaction which delivers the HRP (6, 46, 47). Furthermore, tyramides with different types of haptens (both fluorescent and non-fluorescent) could be used for the signal amplification step. Haptens could be involved in another anti-hapten antibody reaction, and antibodies can again be labeled with HRP for a second (and a third) layer of CARD, allowing an increase in sensitivity of up to 1000-fold (43). However, one should keep in mind that the size of the HRP alone is approximately 40,000 Dalton, and the size of this complex increases increases when linked to other molecules such as oligonucleotides or antibodies. This may again compromise diffusion through cell walls and membranes and may require major adaptations of permeabilization procedures (47).

In summary, we developed and explored a novel FISH protocol based on CARD and FISH with HRP labeled oligonucleotides. This approach permits the detection of small marine bacteria with low ribosome content that are not or only barely detectable with monolabeled probes.

ACKNOWLEDGEMENTS

We thank M. Mussmann for providing sediment samples, G. Gerdts for organizing the North Sea cruise and help during sampling, and the crew of the RV Uthörn. Dr. V.T. Tempranillo is acknowledged for fruitful discussions, and N. Neese for advise on fluorescent staining. This work was supported by the German Ministery of Education and Research (BMBF 01 LC0021/ TP4) and by the Max Planck Society.

REFERENCES

1. **Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F.-O. Glöckner, A. Wille, and R. Psenner.** 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Appl. Environ. Microbiol. **62:**2138-2144.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762-770.
3. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
4. **Amann, R. I., B. Zarda, D. A. Stahl, and K. H. Schleifer.** 1992. Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **58:**3007-11.
5. **Bobrow, M. N., T. D. Harris, K. J. Shaughnessy, and G. J. Litt.** 1989. Catalyzed reporter deposition, a novel method of signal amplification - application to immunoassays. J. Immunol. Methods. **125:**279-285.
6. **Braissant, O., and W. Wahli.** 1998. A simplified in situ hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica. **1:**10-16.
7. **Cottrell, M. T., and D. L. Kirchman.** 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. **66:**5116-5122.
8. **Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434-444.
9. **DeLong, E. F.** 1992. *Archaea* in coastal marine environments. Proc. Natl. Acad. Sci. USA. **89:**5685-5689.
10. **DeLong, E. F., L. T. Taylor, T. L. Marsh, and C. M. Preston.** 1999. Visualization and Enumeration of Marine Planktonic Archaea and Bacteria by Using Polyribonucleotide Probes and Fluorescent In Situ Hybridization. Appl. Environ. Microbiol. **65:**5554-5563.

11. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic strains: Ribosomal RNA-based probes for the identification of single cells. Science. **243:**1360-1363.

12. **Eilers, H., J. Pernthaler, and R. Amann.** 2000. Succession of pelagic marine bacteria during enrichment: A close look on cultivation-induced shifts. Appl. Environ. Microbiol. **66:**4634-4640.

13. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044-3051.

14. **Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann.** 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. **67:**5134-5142.

15. **Fuchs, B. M., F. O. Glockner, J. Wulf, and R. Amann.** 2000. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **66:**3603-3607.

16. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature. **356:**148-149.

17. **Fuhrman, J. A., and C. C. Ouverney.** 1998. Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native *Archaea* with fluorescent single cell probes. Aquatic Ecology. **32:**3-15.

18. **Gasol, J. M., P. A. delGiorgio, R. Massana, and C. M. Duarte.** 1995. Active versus inactive bacteria: size-dependence in a coastal marine plankton community. Mar. Ecol. Prog. Ser. **128:**91-97.

19. **Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K.-H. Schleifer.** 1996. An *in situ* hybridization protocol for detection and identification of planktonic bacteria. Syst. Appl. Microbiol. **19:**403-406.

20. **Glöckner, F. O., B. M. Fuchs, and R. Amann.** 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. **65:**3721-3726.

21. **Glöckner, F.-O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Pernthaler, and R. Amann.** 2000. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of Actinobacteria. Appl. Environ. Microbiol. **66:**5053-5065.

22. **Gonzalez, J. M., R. Simo, R. Massana, J. S. Covert, E. O. Casamayor, C. Pedros-Alio, and M. A. Moran.** 2000. Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. Appl. Environ. Microbiol. **66:**4237-4246.

23. **Heissenberger, A., G. G. Leoppard, and G. J. Herndl.** 1996. Relationship between the intracellular integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. Appl. Environ. Microbiol. **62:**4521-4528.

24. **Hopman, A. H. N., F. C. S. Ramaeker, and E. J. M. Speel.** 1998. Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for in situ hybridization using CARD amplification. J. Histochem. Cytochem. **46:**771-777.

25. **Karner, M., E. F. DeLong, and D. M. Karl.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature. **409:**507-509.

26. **Karner, M., and J. A. Fuhrman.** 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl. Environ. Microbiol. **63:**1208-1213.

27. **Lebaron, P., P. Catala, C. Fajon, F. Joux, J. Baudart, and L. Bernard.** 1997. A new sensitive, whole-cell hybridization technique for detection of bacteria involving a biotinylated oligonucleotide probe targeting rRNA and tyramide signal amplification. Appl. Environ. Microbiol. **63:**3274-3278.

28. **Lebaron, P., P. Servais, H. Agogue, C. Courties, and F. Joux.** 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Appl. Environ. Microbiol. **67:**1775-1782.

29. **Lee, S. H., C. Malone, and P. F. Kemp.** 1993. Use of multiple 16s ribosomal-rRNA-targeted fluorescent-probes to increase signal strength and measure cellular rRNA from natural planktonic bacteria. Marine Ecology-Progress Series. **101:**193-201.

30. **Llobet-Brossa, E., R. Rossellò-Mora, and R. Amann.** 1998. Microbial community composition of Wadden Sea sediments as revealed by Fluorescence-*in-situ*-Hybridization. Appl. Environ. Microbiol. **64:**2691-2696.

31. **Morita, R. Y.** 1997. Bacteria in oligotrophic environments: starvation-survival lifestyle, vol. 1. Chapman Hall, New York.

32. **Mullins, T. D., T. B. Britschgi, R. L. Krest, and S. J. Giovannoni.** 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol. Oceanogr. **40:**148-158.

33. **Murray, A. E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge, and E. F. DeLong.** 1999. A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. Aquat. Microb. Ecol. **20:**129-145.

34. **Oda, Y., S. J. Slagman, W. G. Meijer, L. J. Forney, and J. C. Gottschal.** 2000. Influence of growth rate and starvation on fluorescent in situ hybridization of *Rhodopseudomonas palustris*. FEMS Microbiol. Ecol. **32:**205-213.

35. **Ouverney, C. C., and J. A. Fuhrman.** 1997. Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Appl. Environ. Microbiol. **63:**2735-2740.

36. **Pernthaler, A., C. M. Preston, J. Pernthaler, E. F. DeLong, and R. Amann.** 2002. A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Appl. Environ. Microbiol.:*in press*.

37. **Pernthaler, J., F. O. Glöckner, W. Schönhuber, and R. Amann.** 2001. Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, p. 207-226. *In* J. H. Paul (ed.), Methods In Microbiology, vol. 30. Academic Press, San Diego.

38. **Rappe, M. S., D. A. Gordon, K. L. Vergin, and S. J. Giovannoni.** 1999. Phylogeny of actinobacteria small subunit (SSU) rRNA gene clones recovered from marine bacterioplankton. Syst. Appl. Microbiol. **22:**106-112.

39. **Ravenschlag, K., K. Sahm, and R. Amann.** 2001. Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). Appl. Environ. Microbiol. **67:**387-395.

40. **Schönhuber, W., B. Fuchs, S. Juretschko, and R. Amann.** 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol. **63:**3268-3273.

41. **Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig, and R. Amann.** 1999. In situ identification of cyanobacteria with horseraddish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **65:**1259-1267.

42. **Simon, M., F. O. Glöckner, and R. Amann.** 1999. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. Aquat. Microb. Ecol. **18:**275-284.

43. **Speel, E. J. M., A. H. N. Hopman, and P. Komminoth.** 1999. Amplification methods to increase the sensitivity of in situ hybridization: Play CARD(S). J. Histochem. Cytochem. **47:**281-288.

44. **Suzuki, M. T., C. M. Preston, F. P. Chavez, and E. F. DeLong.** 2001. Quantitative mapping of bacterioplankton populations in seawater: field tests across an upwelling plume in Monterey Bay. Aquat. Microb. Ecol. **24:**117-127.

45. **Suzuki, M. T., L. T. Taylor, and E. F. DeLong.** 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. Appl. Environ. Microbiol. **66:**4605-4614.

46. **van de Corput, M. P. C., R. W. Dirks, R. P. M. van Gijlswijk, F. M. van de Rijke, and A. K. Raap.** 1998. Fluorescence in situ hybridization using horseradish peroxidase-labeled oligodeoxynucleotides and tyramide signal amplification for sensitive DNA and mRNA detection. Histochem. Cell Biol. **110:**431-437.

47. **Wagner, M., M. Schmid, S. Juretschko, K. H. Trebesius, A. Bubert, W. Goebel, and K. H. Schleifer.** 1998. In situ detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*. FEMS Microbiol. Lett. **160:**159-168.

48. **Yang, H., I. B. Wanner, S. D. Roper, and N. Chaudhari.** 1999. An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. J. Histochem. Cytochem. **47:**431-445.

49. **Zaitsu, K., and Y. Ohkura.** 1980. New fluorogenic substrates for horseradish-peroxidase - rapid and sensitive assays for hydrogen-peroxide and the peroxidase. Anal. Biochem. **109:**109-113.

50. **Zubkov, M. V., B. M. Fuchs, S. D. Archer, R. P. Kiene, R. Amann, and P. A. Burkill.** 2001. Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulfoniopropionate in an algal bloom in the North Sea. Environ. Microbiol. **3:**304-311.

4

Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton

Annelie Pernthaler, Jakob Pernthaler, Martha Schattenhofer, and Rudolf Amann.

Submitted.

Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton

Annelie Pernthaler, Jakob Pernthaler, Martha Schattenhofer, and Rudolf Amann

Molecular Ecology Group, Max-Planck-Institute for Marine Microbiology, Bremen, Germany

We present a method for the microscopic identification of DNA synthesizing cells in bacterioplankton samples. After incubation with the halogenated thymidine analogon bromodeoxyuridin (BrdU), environmental bacteria were identified by fluorescence in situ hybridization (FISH) with horseradish peroxidase (HRP) linked oligonucleotide probes. Tyramide signal amplification (TSA) was used to preserve the FISH staining during the subsequent immunocytochemical detection of BrdU incorporation. DNA-synthesizing cells were visualized by means of a HRP-labeled antibody fab fragment and a second TSA step. We applied our protocol to samples of 1.2 μm-prefiltered North Sea surface water collected during early autumn. After 4 h of incubation BrdU-incorporation was detected in 3% of all bacterial cells. Within 20 h the detectably DNA-synthesizing fraction increased to >14%. During this period, the abundances of members of the Roseobacter lineage remained constant, but the fraction of BrdU-incorporating Roseobacter sp. cells doubled, from 24% to 42%. In Alteromonas sp. high BrdU labeling rates after 4-8 h were followed by a 10fold increase in abundances. Rapid BrdU incorporation was also observed in members of the SAR86 lineage. After 4 h of incubation cells affiliated with this clade constituted 8% of total abundances, but almost 50% of the visibly DNA-synthesizing bacterial fraction. This clade might thus be an important contributor to total bacterioplankton activity during periods of low primary production. The small cell size and low ribosome content of SAR86 cells is probably no indication of inactivity or dormancy.

As the awareness of the importance, diversity, and complexity of marine microbial communities has developed, it is increasingly recognized that an understanding of the ecology of the various bacterial (and archaeal) populations requires simultaneous information both about the identity and the activity of individual cells in environmental samples (8, 27, 49). Consequently, a considerable amount of recent research effort has focused on the development of techniques that permit the estimation of various activities of single bacteria cells (16, 35). In addition, cultivation-independent approaches for the quantification of population sizes of individual bacterial taxa have been developed , such as fluorescence in situ hybridization (FISH) with oligonucleotide or polynucleotide rRNA-targeted probes (1, 10, 17). FISH is an increasingly popular tool for the microscopic visualization of individual groups of bacteria and archaea in the marine environment (7, 9, 14, 22, 29, 31). Recent technical advances have furthermore greatly increased the sensitivity of this staining technique (9, 29, 30), so that the low ribosome content of many planktonic bacteria no longer limits the applicability of FISH to productive or coastal regions (30). One of the challenges of present-day microbial ecology is to combine FISH with techniques that allow to determine defined microbial activities, e.g. with microautoradiography (8).

The introduction of tritiated thymidine (TdR) as a tracer has provided the possibility to estimate both community DNA synthesis rates by bulk uptake measurements (15) and the fraction of TdR-incorporating cells by microautoradiography (5, 28, 41). Bromodeoxyuridine (BrdU) is a halogenated nucleotide analogue of TdR that is also incorporated into newly synthesized DNA. Pulse-labeling of DNA with BrdU and subsequent immunocytochemical detection (ICC) of labeled DNA is extensively used in histochemistry and cytochemistry to study the proliferation of eucaryotic cells (25, 34), whereas this technique has received comparatively little attention in microbial ecology. A keyword search of the Institute of Scientific Information database returns more than 5200 citations containing the term "bromodeoxyuridine" for the past 10 years, but only four of these are relevant to environmental microbiology (4, 43, 45, 48). So far BrdU has been applied as a non-radioactive alternative to TdR to measure bacterioplankton growth rate (43), to separate DNA of growing and inactive bacteria (4, 45, 48), and for the immunochemical labeling of marine bacterial isolates in pure cultures (45).

One major drawback of the ICC approach is that presently the available highly sensitive methods for the detection of incorporated BrdU into whole cells cannot be employed for the microscopic visualization of individual bacterial cells from natural

assemblages (38). Cell walls and membranes need to be permeabilized for antibody penetration, which usually causes a high and species specific cell loss (29). In addition, difficulties in cell preparation arise because the anti-BrdU-antibody only reacts with single stranded DNA (25). Here we present a protocol for the immunocytochemical detection of incorporated BrdU into the DNA of single bacterioplankton cells, and the simultaneous identification of individual DNA-synthesizing populations by fluorescence in situ hybridization (FISH). To increase the sensitivity of the techniques, both FISH and BrdU-detection were coupled with signal amplification by catalyzed reporter deposition (CARD) (29, 42).

MATERIALS AND METHODS

Study site, sample preparation, bacterial abundances. Water samples were taken on September 24, 2001 from 1 m depth at the sampling site Helgoland Roads (54°09'N 7°52'E) near the island of Helgoland, which is situated 23 miles offshore in the German Bay of the North Sea. Sea water was prefiltered through 1.2 μm cellulose nitrate membrane filters (Sartorius, Göttingen, Germany) within 1 hour after sampling, and subsamples (700 ml) were filled in acid-prewashed glass bottles. The filtrates were either supplemented with 5-bromo-2'-deoxy-uridine (20 μM, Roche Diagnostics, Mannheim, Germany) and TdR (33 nM, Sigma-Aldrich, Seelze, Germany); or only TdR as controls. The addition of 33 nM TdR was done to block the de novo synthesis of thymidine (45). Treatments were incubated in duplicates in the dark at in situ temperature (10 °C) and mild agitation. Subsamples were taken at 4 timepoints (0, 4, 8, 20 h) and fixed at 4°C for 20 hours in particle-free formaldehyde solution (final concentration, 2% volume/volume, v/v). For FISH and ICC, portions of 50 ml were filtered onto white polycarbonate membrane filters (type GTTP, pore size, 0.2 μm, size, 47 mm, Millipore, Eschborn, Germany), washed with 10 ml of deionized, particle-free water (MQ), and stored at –20°C until further processing. Bacterial total abundances were determined by epifluorescence microscopy after staining with 4',6'-diamidino-2-phenylindol (DAPI).

Bacterial isolates, growth conditions, and postincubation processing. Stationary phase cultures of 20 marine bacterial isolates (Table 1) were inoculated into 10 ml of autoclaved North Sea water amended with 0.05 g of yeast extract. The liquid cultures were supplemented with TdR (33 nM) and BrdU (20 μM), and grown at room temperature (RT) in the dark for 21 h. Negative controls without BrdU were produced for each strain. The isolates were affiliated with the gamma proteobacteria, the *Cytophaga-Flavobacterium*

group and the *Actinobacteria*. A stationary phase culture of *Escherichia coli* was transferred to fresh Luria Broth (LB) medium amended with nalidixic acid [50 mg l^{-1}] to block DNA de novo synthesis, and incubated at 37°C. Control incubations were performed on LB medium without nalidixic acid. 30 min after inoculation cultures were pulsed with BrdU and TdR for 1.5 h and subsequently fixed as described above. Subsamples (0.1 - 5 ml) of the cell suspensions were filtered onto white polycarbonate membrane filters (type GTTP, pore size, 0.2 μm, size, 25 mm, Millipore, Eschborn, Germany), washed with 10 ml of MQ, and stored at –20°C until further processing.

Table 1. Bacterial strains that were tested for their ability to incorporate BrdU

strain number	accession number	next relative (16S rDNA)	group[1)]	labelling index
0234A	AF235124	*Flavobacterium columnare*		> 90%
02ds22	AF235114	*Cytophaga uliginosa*	CFB	100%
11ds02	AF235111	*Cytophaga marinoflava*	clade	100%
0803	AF235117	*Flavobacterium salegense*		100%
11ds10	AF239707	NOR6 clade		100%
KT71	AY007676	NOR5 clade		> 90%
0246	AF173966	*Shewanella* sp.		100%
0919	AF173964	*Colwellia psychrophila*		> 90%
0903	AF235119	*Pseudoalteromonas atlantica*		100%
0910	AF173963	*Pseudoalteromonas halopla.*		100%
KT15	-	*Polaribacter* sp.		80-90%
0924	-	*Oceanospirillum* sp.		> 90%
0232	AF235125	*Alteromonas* sp.		> 90%
1111	AF173968	*Halomonas* sp.		> 90%
0901	AF172840	Vibrio splendidius		> 90%
0248	AF235127	*Photobacterium*		100%
11ds07	AF235112	NOR1 clade		100%
1114	AF235108	NOR2 clade		100%
1115	AF2315113	*Micrococcus* sp.	*Actino-*	30-40%
1110	AF239706	NOR7 clade	*bacteria*	100%
ATCC11775^T	-	*Escherichia coli*		100%

1) CFB, Cytophaga – Flavobacterium – Bacteroides

Sample preparation for FISH and ICC. A detailed protocol of all steps of sample processing for CARD-FISH and ICC is given in Table 2. To avoid cell loss during cell wall permeabilization and epitope retrieval, filters were dipped in low gelling point agarose (0.2% [weight/volume, w/v] in MQ, MethaPhor, Bioproducts, Rockland, Maine), dried on glass slides at 46°C and subsequently dehydrated in 96% [v/v] ethanol for 1 min (29, 37).

Table 2. Summary of steps for FISH & BrdU detection in marine bacteria

Embedding	prepare subsamples on membrane filters
	dip filter in 0.2% low gelling point agarose, place filters face up onto glass slides and let air dry at 46°C
	dehydrate in 96% ethanol (1 min, room temperature [RT]), air dry [1)]
Permeabilization and epitope unmasking	incubate in epitope retrieval buffer (60°C, 15 min), wash in MQ
	wash in HCl [1M] (60°C, 5 seconds), wash in MQ
	incubate in permeabilization buffer (60°C, 5 min),
	wash in MQ, wash in 96% ethanol, let air dry
	repeat embedding step
	incubate with Lysozyme [10 mg/ml, 0.1M Tris, 0.05M EDTA] (37°C, 60 min)
	wash in MQ, wash in 96% ethanol, air dry [1)]
Hybridization	place sections in reaction vial (1.5 ml, 10 – 20 sections per vial) & cover filter sections with 1000 μl of hybridization buffer [0.5 ng μl^{-1} probe]
	incubate at 35°C for 2 hours
	wash filters in prewarmed washing buffer (5 min, 37°C)
Tyramide signal amplification	incubate in PBST (50 ml, RT, 15 min)
	dab filter on blotting paper, but don`t let run dry
	incubate in substrate mix [1 part $Alexa_{488}$-tyramide, 100 parts amplification buffer] (37°C, 10 min, in the dark)
	dab filter on blotting paper
	wash in: 50 ml PBST (RT, 5 min, in the dark), 10 ml MQ (RT, 1 min), 10 ml 96% ethanol (RT, 1 min), air dry [1)]
Antibodyreaction	bleach peroxidases with 0.01 M HCl (RT, 10 min), wash 3 times in excess MQ
	place sections on parafilm & cover with 500 μl of Anti-BrdU reaction mix [1.5 U/ml Anti-BrdU-HRP, 1x PBS, 1% Blocking Reagent, 20 U ml^{-1} HAE III], seal with parafim & incubate at 37°C for 2.5 - 3 hours
	wash filters in 50 ml PBST (10 min, RT)
Tyramide signal amplification	dab filter on blotting but, don`t let run dry
	incubate in substrate mix [1 part $Alexa_{536}$-tyramide, 200 parts amplification buffer] (37°C, 10 min, in the dark)
	dab filter on blotting paper, wash in: 50 ml PBST (2 min, RT), 10 ml MQ (RT, 1 min), 10 ml 96% ethanol (RT, 1 min), air dry [1)]
	samples can now be embedded and visualized

[1)] preparations may be stored at –20°C for several days to weeks without apparent loss in signal

For DNA denaturation the filters were (a) incubated in preheated epitope retrieval buffer (95% formamide [v/v], 1x SSC [15 mM sodium citrate, 150 mM sodium chloride, pH 7.0], 0.5% [v/v] Triton X-100) at 60°C for 15 min and subsequently washed 3 times in excess MQ at room temperature (RT), (b) dipped for 5 seconds in preheated HCl (1 M, 60°C) and then washed 3 times in excess MQ at RT, and (c) incubated in prewarmed permeabilization buffer (0.1 M TrisHCl, 0.1 M EDTA, 1% [v/v] Triton X-100) at 60°C for 5 min for non-enzymatic permeabilization , then washed in MQ at RT, dehydrated in 96% [v/v] ethanol for 1 min and air dried. Next, a second agarose embedding step was performed as described above. The air dried and double-embedded filters were subsequently incubated in a lysozyme solution (10 mg/ml, 0.1M Tris, 0.05M EDTA) at 37°C for 60 min, then washed in MQ, dehydrated in 96% [v/v] ethanol for 1 min and air dried.

Synthesis of fluorescently labeled tyramides. TyramideHCl (Fluka, Taufkirchen, Germany) was labeled with succinimidyl esters of the fluorescent dyes $Alexa_{488}$, $Alexa_{546}$, and $Alexa_{350}$ (Molecular Probes, Leiden, The Netherlands) as has been described for other fluorophores (21). Tyramide stock solution was prepared by dissolving 10 mg tyramideHCl in 1 ml of dimethylformamide (DMF), containing 10 μl triethylamine. Active dye esters were dissolved in DMF (10 mg ml^{-1}) and were added in 1.1-fold equimolar amounts to the tyramide stock solution and incubated at RT in the dark for 2 h on a rotation shaker (10 rpm). All stock solutions had to be freshly prepared (< 10 min) prior to the labeling reaction. The synthesized tyramide conjugates were diluted with absolute ethanol to obtain a concentration of 1 mg ml^{-1}. Desiccated aliquots were stored at –20°C. For signal amplification reaction, conjugated tyramides were freshly dissolved in DMF and stored at –20°C for at least 2 months without any reduction in reactivity.

FISH with enzyme-labeled oligonucleotide probes. Up to 25 individual filter sections were put into a 1.5 ml reaction vial, and covered with 1000 μl of hybridization buffer (0.9 M NaCl, 20 mM TrisHCl [pH 7.5], 10% dextrane sulfate [w/v], 0.02% [w/v] sodium dodecyl sulfate [SDS], 55% [v/v] formamide [Fluka], 1% [w/v] Blocking Reagent [Boehringer, Mannheim, Germany], horseradish peroxidase (HRP) labeled oligonucleotide probes [Interactiva, Ulm, Germany; final concentration, 0.5 ng μl^{-1}] prepared as described previously (29)). The vial was incubated at 35°C for 2 hours on a rotation shaker (10 rpm). Next the filter sections were removed from the hybridization mixture, and incubated in 50 ml of prewarmed washing buffer (3 mM NaCl, 5 mM EDTA [pH 8.0], 20 mM TrisHCl [pH 7.5], 0.01% [w/v] SDS) at 37°C for 5 min. The FISH probes were targeted to *Bacteria*

(EUB338), *Roseobacter* sp. (ROS537) (14), the SAR86 clade (SAR86-1249), *Oceanospirillum* sp. (OCE232), *Alteromonas* sp. (ALT1413), and *Pseudoalteromonas* sp. (PSA184) (12).

In situ detection of HRP-conjugated oligonucleotide probes. To equilibrate the probe-delivered HRP, sections were put into 50 ml of PBST (1× phosphate buffered saline, 0.05% of Triton X-100) for 10 to 15 min at room temperature. To remove excess buffer, the filter sections were dabbed onto blotting paper, and immediately transferred to a substrate mix containing 1 part of tyramide-$Alexa_{488}$ (or $Alexa_{350}$ for isolates) and 100 parts of amplification buffer (1 x PBS, 0.0015% H_2O_2), and incubated for 10 to 15 min at 37°C in the dark. Filter sections were again briefly put on blotting paper and washed for 5-10 min at room temperature in the dark in 50 ml of PBST. To decrease background fluorescence, sections were subsequently washed in MQ and 96% ethanol for 1 min each. At this stage, the preparations were either embedded in DAPI-amended mountant for FISH counts (see below), or further processed for BrdU detection.

Immunocytochemical detection of incorporated BrdU. In order to bleach the probe delivered peroxidases the filters were incubated in 0.01 M HCl for 10 min at RT in the dark, and subsequently washed 3 times in excess MQ. Filter sections were then placed onto parafilm, and covered with 500 μl of antibody mixture (Anti-BrdU-HRP, Fab fragments, clone BMG-6H8 [1.5 U ml^{-1}, Roche Diagnostics, Mannheim, Germany], 1 x PBS, 1 % [w/v] Blocking Reagent, DNA restriction enzyme [*HAE* III, 20 U ml^{-1}, New England Biolabs, Beverly, MA]). The samples were then sealed with parafilm, placed in a petri dish and incubated at 37°C for 2.5 - 3 h, and subsequently washed for 10 min at RT in 50 ml of PBST. Signal amplification (1 part tyramide-$Alexa_{546}$, 200 parts amplification buffer) was carried out as described above for FISH. Filters were then air dried and stored at –20°C until further processing (within 3 days)

Microscopic evaluation. Filter sections were covered in mountant (11 parts of Citifluor AF1 [Citifluor Ltd., London, U.K.], 2 parts of VectaShield [Vector Laboratories, Burlingame, CA], 1 part of 1 x PBS, amended with DAPI [final concentration 1 μg / μl]). Samples were evaluated on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany), equipped with a HBO 100 W Hg vapor lamp, appropiate filter sets for Cy3 ($Alexa_{546}$), FITC ($Alexa_{488}$) and DAPI ($Alexa_{350}$) fluorescence (18), and a 100x Plan Apochromat objective. Between 600 and 800 DAPI stained objects per filter were counted in hybridized sample without ICC staining. In double-stained FISH and ICC preparations the fraction of

BrdU-incorporating cells of all hybridized cells were quantified from 10 individual microscopic fields.

RESULTS

Optimization of sample pretreatment and ICC. Since heat (39), DNase (11, 44), detergents (47) and HCl (2) are all known to be useful in recovering immunoreactivity of BrdU epitopes in formalin fixed tissues and cell preparations, it was required to investigate the effects of a variety of agents in combination with each other and at different temperatures on BrdU cell labeling indices. Best results (highest labeling indices) were achieved by a combination of several permeabilizing and epitope unmasking steps (Table 2). Epitope retrieval was performed before hybridization, because of potential FISH signal loss during treatment with hot acid and formamide. In addition, samples were incubated with lysozyme, as is required for CARD-FISH permeabilization (29, 37). Filters that were not treated for epitope retrieval produced only very few Anti-BrdU-HRP signals.

Although BrdU incorporated into DNA was readily detected after heat, formamide and acid treatment only, repeated stainings with Anti-BrdU-HRP differed in signal intensities and labeling indices (data not shown) (33). Preliminary results furthermore suggested that the additional use of DNase I before the antibody reaction sometimes caused substantial loss of the target DNA. Therefore we choosed a restriction enzyme for the relaxation of the denatured DNA. Since no restriction enzymes could be applied that specifically recognize T-containing sites, a GG/CC cutter (*HAE* III) was chosen (32). Nuclease digestion was carried out simultaneously with antibody binding. This strategy has been previously employed (11, 43) and is the recommended protocol in some commercially available cell proliferation assays (Boehringer Mannheim). For the subsequent quantification of labeling indices of isolates, and of DNA-synthesizing North Sea bacterioplankton populations by FISH and ICC, we used the optimal set of conditions as determined in our preceeding experiments (Table 2).

We also investigated if the various permeabilization procedures would result in loss of cells from the filters. Initially, we sometimes observed detachment of pieces of the agarose cover after the lysozyme incubation, possibly caused by acid and heat during epitope retrieval. Therefore the embedding step was repeated before treatment with lysozyme. This effectively restored the protective properties of the agarose cover during all subsequent incubations. The mean bacterial cell numbers (DAPI counts) in the bacterioplankton samples were $5.4 \pm 0.6 \times 10^5$ cell ml^{-1} (mean ± 1 standard deviation) in

the untreated samples, and 5.1 ± 0.6 x 10^5 cells ml^{-1} after all permeabilization steps. In summary, no significant decrease was observed as a result of the pretreatments (Wilcoxon matched pair test, n = 10, p > 0.05).

Bacterial isolates. All investigated strains including *E. coli* were able to incorporate BrdU into DNA (Table 1). Because the DAPI-signal is partially lost during the ICC staining, likely due to the presence of the restriction enzyme in the antibody reaction buffer, the relative abundances of active bacteria were always determined as the fraction of cells hybridized with EUB338-HRP (% EUB). The majority of bacterial cultures that had been supplemented with BrdU showed labeling indices above 90% EUB. Using our protocol, we did not detect Anti-BrdU-HRP positive cells in samples that were not supplemented with BrdU. In BrdU pulse labeled *E. coli* cultures we observed positive staining of cells with Anti-BrdU-HRP only in those samples without addition of nalidixic acid.

Quantification of bacterioplankton by FISH and ICC. During 20 hours of incubation the total cell numbers as determined by DAPI counts did not change or increased very little in the various treatments (Fig. 1).

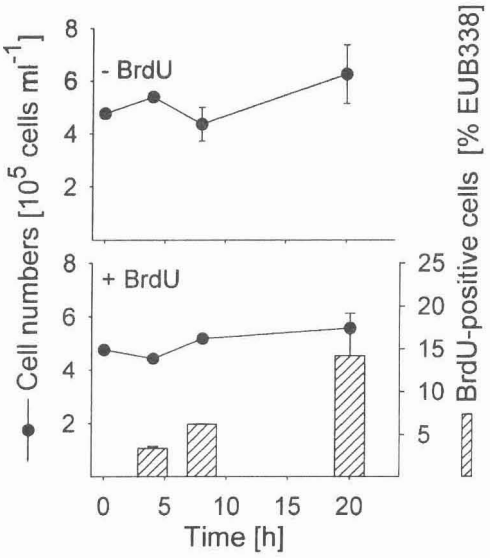


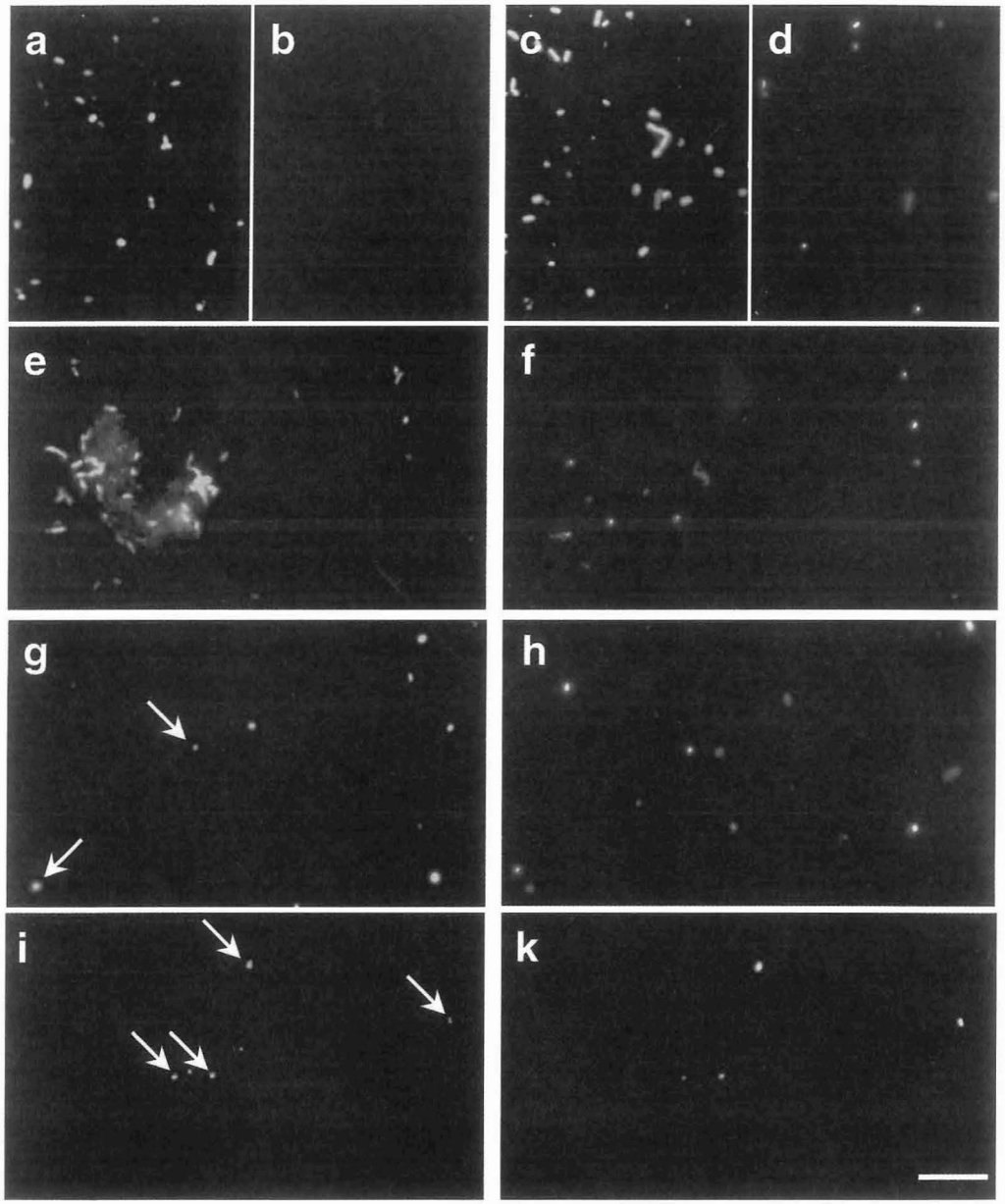
Figure 1. Lines & symbols: Abundances of bacterioplankton cells in 1.2 μm filtrates of North Sea surface water detected by probe EUB338-HRP during 20 h of incubation. Bars: Percentage of Anti-BrdU-HRP positive *Bacteria*. Errorbars indicate total ranges of duplicates.

Bacterial cells visualized by FISH with EUB338-HRP accounted for the majority (mean 97.9%; range: 90 - 100%) of DAPI stained cells (%DAPI) (table 3). Therefore, it was adequate to determine the total abundances of BrdU-positive bacteria as the fractions of all hybridized cells (%EUB). Cells hybridizing with probes OCE232 and PSA184 were below 1% DAPI and were therefore excluded from further analysis.

Table 3. Detection rates of probe EUB338-HRP in filtrates and incubations of North Sea surface water

	- BrdU		+ BrdU	
	I	II	I	II
t_{0h}	94.13	94.13	94.14	94.14
t_{4h}	90.42	98.66	93.69	99.21
t_{8h}	93.47	99.62	97.68	99.25
t_{20h}	100.00	100.00	100.00	100.00

In all three studied bacterial populations BrdU-incorporating cells could be readily visualized by BrdU antibody staining and CARD (Fig. 2). Total detection rates with the anti-BrdU-HRP antibody increased from 3.4% EUB (range, 3.2 – 3.6%) after 4 h of incubation to 14.2% EUB (9.3 – 19.2%) after 20 h (Fig. 1). We found no anti-BrdU-HRP positive cells in the t_0 sample and in samples that were not supplemented with BrdU. Bacteria affiliated with *Roseobacter* showed no clear change in cell numbers during 20 h of incubation (Fig. 3), and the detection rates of probe ROS537-HRP ranged between 1.4 to 3.3% DAPI (mean, 2.4%). BrdU labeling indices of *Roseobacter* increased from 24% of cells detected with probe ROS537 (20.6 – 28.1%) after 4 h of incubation to 43% (32.3 – 53.0). Cell numbers of *Alteromonas* sp. increased by 10fold during 20 h of incubation, from 5,200 to 49,000 cells ml^{-1} (Fig. 4). Although the relative abundances of cells hybridizing with probe ALT1413 rose from initially 1% to 9% DAPI (range, 8.1 – 9.5%), this change was too small to be clearly reflected in the total bacterial cell numbers (Fig. 1). The percentages of Anti-BrdU-HRP positive cells were highest (mean, 43.6% ALT; range, 36 – 51%) at the onset of growth after 8 h of incubation, and declined thereafter (6.3% ALT, 5.4 – 7.1%). Members of the SAR86 clade constituted 8.1% of the total cell counts, but represented almost 50% of all DNA synthesizing bacteria after 4 h of BrdU incubation.



Photomicrographs of FISH and Anti-BrdU-HRP stained marine bacteria. Each double panel depicts FISH signal in green (left) and Anti-BrdU-HRP signal in red (right). Panels (a) and (b) probe EUB338-HRP and Anti-BrdU-HRP staining in incubations without BrdU; (c) and (d) probe EUB338-HRP and Anti-BrdU-HRP staining in incubations supplemented with BrdU; (e) and (f) probe ALT1413-HRP, specific for members of the *Alteromonas* lineage, and Anti-BrdU-HRP staining, (g) and (h) probe ROS537-HRP, specific for members of the *Roseobacter* lineage, and Anti-BrdU-HRP staining, (i) and (k) probe SAR86-1249-HRP, specific for members of the SAR86 clade, and Anti-BrdU-HRP staining. Scale bar: 10 μm.

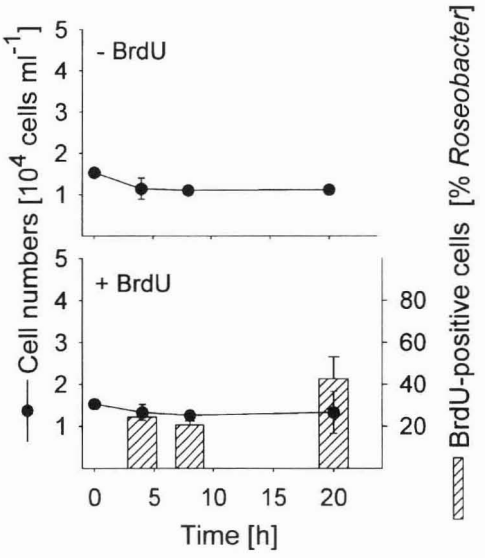


Figure 3. Lines & symbols: Abundances of members of the *Roseobacter* lineage detected by probe ROS537-HRP. Bars: Percentage of Anti-BrdU-HRP positive *Roseobacter*. Errorbars indicate total ranges of duplicates.

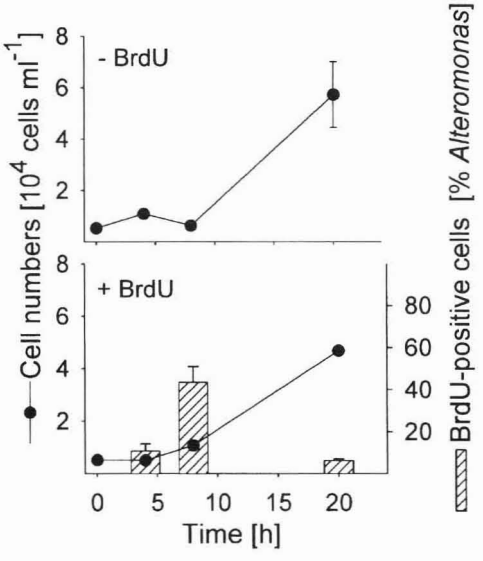


Figure 4. Lines & symbols: Abundances of members of the *Alteromonas* lineage detected by probe ALT1413-HRP. Bars: Percentage of Anti-BrdU-HRP positive *Alteromonas*. Errorbars indicate total ranges of duplicates.

During the incubations SAR86 decreased in abundances from 3.8 x 10^4 cells ml^{-1} at t_0 to 2.2 x 10^4 cells ml^{-1}. (2.0 – 3.2 x 10^4) (Fig. 5). Concomitantly, a decline in FISH intensities of hybridized cells was observed after 20 h despite CARD signal amplification. The percentage of Anti-BrdU-HRP positive SAR86 were highest after 8 h of incubation (40.2% SAR86, 37 – 44%) and remained constant or declined even slightly (mean: 31.7, range: 26 – 38% SAR86) after 20 h.

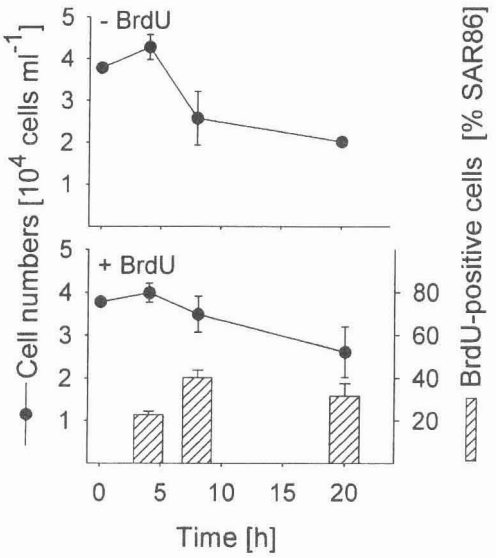


Figure 5. Lines & symbols: Abundances of members of the SAR86 clade detected by probe SAR86-1249-HRP. Bars: Percentage of Anti-BrdU-HRP positive SAR86. Errorbars indicate total ranges of duplicates.

DISCUSSION

We successfully developed a protocol for the immunocytochemical detection of BrdU incorporation into whole bacterial cells from environmental samples. In spite of low ambient temperatures (10°C) and the typically low DNA synthesis rates of slowly growing bacterioplankton during autumn (40) a population of BrdU-positive cells could be readily visualized after 4 h of incubation (Fig. 1). Similar estimates of the DNA-synthesizing fraction of free-living bacterioplankton cells were obtained by microautoradiography of tritiated TdR ([^{3}H]TdR) (28). It is nevertheless possible that only the most rapidly DNA-synthesizing bacteria are visualized by our method. We can envisage two potential

shortcomings of the ICC detection of DNA synthesis: (a) the signal intensity sets a threshold to detection and (b) not all pelagic bacteria are able to incorporate BrdU.

Sensitivity of the BrdU assay. CARD amplification of the BrdU signal was required to reduce the risk of underestimating the number of proliferating cells. The use of HRP labeled antibodies for the detection of BrdU results in a significant increase of signal intensity as compared to the weak and heterogenous signal of directly fluorochrome labeled antibodies (46). To our knowledge, this is the first report of using a directly HRP-labeled antibody Fab fragment for the immunocytochemical staining of BrdU. Such a fragment is probably more suitable for antigen detection inside bacterial cells than a whole antibody because of its smaller size (50 kDa, as compared to approximately 160 kDa of a whole IgG antibody).

BrdU labeling indices are affected by many factors and incorporation rates can be underestimated, depending on the choice of fixative and/or pretreatment (24). For example, it has been shown that the influence of formalin on tissue antigenicity depends on the total time of fixation. This means that immunostaining results can only be compared when the fixative, the time of fixation and the way of epitope retrieval are standardized. In our experiment, we used formaldehyde fixation, since to our knowledge, this is the only strategy which is useful if a subsequent permeabilization with lysozyme is required. Massive cell loss was observed after lysozyme treatment if samples were fixed with ethanol (A.P., unpubl. data).

Selective uptake and potential toxicity of BrdU. Twenty years ago, microbiologists claimed that wild-type bacteria do not take up BrdU to any significant extent at all (6). However, the *E. coli* strain, and all 20 tested marine bacterial isolates were able to incorporate BrdU into DNA (Table 1). The majority of the isolates showed BrdU labeling indices >90% EUB, indicating that our protocoll is adequate for the detection of growth in a variety of marine bacteria. In contrast to a previous report (45), several isolates from the *Cytophaga – Flavobacterium* lineage could also be readily stained by our assay. This finding is important, because a large fraction of the bacterioplankton in coastal North Sea surface waters is formed by *Cytophaga*-related microbes during the spring and summer months (13). Judging from our results both on isolates and on uncultured populations (table 1, Figs. 3, 5), it is thus likely that numerous members of the marine bacterioplankton will be able to take up and incorporate BrdU.

Several assumptions underlie the use of TdR and BrdU as tracer for the study of DNA synthesis patterns in cell populations (38). For example, it is assumed that [^{3}H]TdR

is either incorporated into DNA by cells or lost, i.e., that the tritium label is not transferred to cellular constituents other than DNA as a result of thymidine catabolism. It is also assumed that cells do not have large endogenous thymidine pools, and will thus incorporate exogenous [^{3}H]TdR into DNA if the labeled material is present during the period of DNA synthesis. One advantage of BrdU as compared to [^{3}H]TdR is its likely greater stability and perhaps greater specificity: During immunodetection, the specificity of the antibody for BrdU would presumably ensure that brominated degradation products, whether produced during storage or by cell metabolism, would not be detected if incorporated into other compounds. Moreover, previous studies have established that TdR added to the samples at trace concentrations [33 nM] is sufficient to inhibit the activity of thymidylate synthase, an enzyme required for de novo synthesis of thymidine monophosphate, in bacterial strains capable of importing TdR (26). This inhibition forces dependence on imported nucleotide, resulting in increased incorporation of exogenously supplied BrdU.

In many eucaryotic cells BrdU selectively affects processes associated with cell differentiation, but has no significant effects on cell growth or overall RNA and protein synthesis (36). At high concentrations, BrdU negatively affected growth rate, viability, and sporulation, of a thymidine requiring *Bacillus subtilis* strain (3). In contrast, the growth kinetics of marine bacterial cultures treated with BrdU and TdR were found to be similar to those treated with TdR alone (45), suggesting a low level of toxicity for BrdU when used at a concentration of 20 µM. Preferential incorporation of TdR over BrdU has been observed in E. coli (20), but again the effect was found to decline with increasing BrdU : TdR in the medium. In summary, BrdU appears to be adaequate as a marker of DNA synthesis for the pulse-labeling and short-term incubation of bacterioplankton.

Simultaneous in situ identification and activity staining. Although there are several methods for the detection of growth-related features in single cells (16, 23, 35), these techniques are not designed to distinguish between individual active bacterial populations. It is increasingly recognized that such a "black-box" approach needs to be supplemented by simultaneous information on bacterial identity (8). Our method has been specifically developed for the study of growth in different members of the bacterioplankton. Based on CARD signal amplification, this approach was sensitive enough for the detection of DNA-synthesis in cells of the SAR86 clade that could not even be visualized by FISH with monolabeled probes because of their low ribosome content (29).

All investigated groups, *Roseobacter*, *Alteromonas*, and SAR86, were capable of incorporating BrdU, but showed different patterns in abundances and BrdU labeling indices. Members of the *Roseobacter* clade did not change in cell numbers during the 20 h of incubation, but exhibited an approximate doubling in the fraction of BrdU positive cells (Fig.3). Since DNA synthesis occurs before cell division, *Roseobacter* showed a pattern typical for the onset of cell division. The incubation conditions thus resulted in an activation of one or several subpopulations from this lineage. This agrees with findings that some *Roseobacter* sp. and related genera are readily culturable (13, 19).

Members of the Alteromonas lineage also visibly synthesized DNA during the incubations, as indicated by BrdU labeling after 8 h of incubation. The high ICC labeling index at that timepoint and the subsequent rise of Alteromonas abundances (Fig. 4) provides evidence that a large fraction of BrdU positive cells indeed preceeds cell multiplication. Therefore, our assay could be a sensitive measure of the in situ growth potential of bacterial populations. Interestingly, the fraction of anti-BrdU-HRP positive Alteromonas cells were lower during during cell multiplication than during the onset of growth. Some strains of this lineage might have selectively decreased the uptake of BrdU, as has been demonstrated for mutant B. subtilis strains (6), or catabolized the incorporated nucleotide. Moreover BrdU is known to undergo a tautomeric shift with higher frequency than TdR. In that case its normal hydrogen bonding is disrupted and it binds to guanine instead of adenine. In the second round of DNA replication, the polymerase recognizes the mismatch and removes one of the two bases. Thus, due to the potential long-term mutagenicity of BrdU and its eventual removal from de novo synthesized genomes, we suggest that the labeling of DNA with BrdU should be carried out in short-term incubations.

After 4 h of incubation the SAR86 population SAR86 constituted 8.1% of total bacterial abundances, but approximatly 50% of all DNA synthesizing bacteria. This indicates that during a season of generally declining bacterial activity (13) SAR86 may represent an important component of the active fraction of the bacterioplankton in coastal North Sea surface waters. It also illustrates that a small cell size and low ribosome content (12) does not imply non-growth or dormancy (16) in this bacterial lineage. Although the SAR86 population was active in the original filtrates, it decreased in cell numbers and in signal intensities of hybridized cells during the incubation (Fig.5). This indicates that SAR86 was deactivated by the treatments. Proteorhodopsin genes have been described from environmental genome fragments of marine bacteria related to SAR86, implying the

potential for light-driven energy generation in this lineage. Since incubations were carried out in the dark (to avoid BrdU phototoxicity), the enrichment conditions might have been detrimental for SAR86 cells. A possible phototrophy of SAR86 would furthermore agree with our finding that this group constituted an important fraction of all DNA-synthesizing cells during a season of low phytoplankton primary production.

While BrdU incorporation in combination with FISH can indicate that specific populations of bacteria in the plankton are growing, this method can not exclude DNA synthesis in a particular species in the environment, unless it has been shown to incorporate BrdU in pure culture. Nevertheless, our approach could provide information about the growth activity of different uncultured microbial populations even during a period when microbial activity is expected to be low. We thus hope that the combination of in situ identification and detection of DNA-synthesis in single cells may allow new insight into the ecological role of different bacterial lineages in the marine plankton.

ACKNOWLEDGEMENTS

The Biologische Anstalt Helgoland is acknowledged for providing guest research facilities. We thank N. Neese for advice on fluorescent staining. This work was supported by the German Ministery of Education and Research (BMBF 01 LC0021/ TP4) and by the Max Planck Society.

REFERENCES

1. Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology. 172(2):762-770.
2. Bak, P. M., and R. J. Panos. 1997. Protease antigen recovery decreases the specificity of bromodeoxyuridine detection in formalin-fixed tissue. Journal of Histochemistry & Cytochemistry. 45(8):1165-1170.
3. Binnie, C., and J. G. Coote. 1986. Inhibition of sporulation in *Bacillus subtilis* by bromodeoxyuridine and the effect on DNA replication. J General Microbiology. 132:493-502.
4. Borneman, J. 1999. Culture-independent identification of microorganisms that respond to specified stimuli. Applied & Environmental Microbiology. 65(8):3398-3400.
5. Brock, T. D. 1967. Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science. 155:81-83.
6. Coote, J. G., and C. Binnie. 1986. Tolerance to bromodeoxyuridine in a thymidine-requiring strain of *Bacillus subtilis*. J General Microbiology. 132:481-492.
7. Cottrell, M. T., and D. L. Kirchman. 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Applied and Environmental Microbiology. 66(12):5116-5122.
8. Cottrell, M. T., and D. L. Kirchman. 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Applied and Environmental Microbiology. 66:1692-1697.
9. DeLong, E., L. Taylor, T. Marsh, and C. Preston. 1999. Visualization and enumeration of marine planktonic *Archaea* and *Bacteria* by using polyribonucleotide probes and fluorescent *in situ* hybridization. Applied and Environmental Microbiology. 65(12):5554-5563.
10. DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. Science. 243:1360-1363.

11. Dinjens, W. N. M., J. Tenkate, M. Lenders, E. P. M. Vanderlinden, and F. T. Bosman. 1992. Bromodeoxyuridine (Brdu) Immunocytochemistry By Exonuclease-Iii (Exo-Iii) Digestion. Histochemistry. 98(3):199-205.

12. Eilers, H., J. Pernthaler, and R. Amann. 2000. Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. Applied and Environmental Microbiology. 66(11):4634-4640.

13. Eilers, H., J. Pernthaler, F. O. Glockner, and R. Amann. 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Applied and Environmental Microbiology. 66(7):3044-3051.

14. Eilers, H., J. Pernthaler, J. Peplies, F. O. Glockner, G. Gerdts, and R. Amann. 2001. Isolation of novel pelagic bacteria from the German bight and their seasonal contributions to surface picoplankton. Applied and Environmental Microbiology. 67(11):5134-5142.

15. Fuhrman, J. A., and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: Evaluation and field results. Mar. Biol. 66:109-120.

16. Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and A. Hagstrom. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Applied and Environmental Microbiology. 65(10):4475-4483.

17. Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. Journal of Bacteriology. 170(2):720-726.

18. Glöckner, F. O., B. M. Fuchs, and R. Amann. 1999. Bacterioplankton composition in lakes and oceans: a first comparison based on fluorescence in situ hybridization. Applied and Environmental Microbiology. 65(8):3721-3726.

19. González, J. M., R. P. Kiene, and M. A. Moran. 1999. Transformation of sulfur compounds by an abundant lineage of marine bacteria in the alpha-Subclass of the class Proteobacteria. Applied and Environmental Microbiology. 65(9):3810-3819.

20. Hanawalt, P. C. 1967. Preparation of 5-bromouracil-labeled DNA, p. 702-708. *In* L. Grossman and K. Moldave (ed.), Methods in Enzymology. Academic Press, New York.

21. Hopman, A. H. N., F. C. S. Ramaekers, and E. J. M. Speel. 1998. Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for in situ hybridization using CARD amplification. Journal of Histochemistry & Cytochemistry. 46(6):771-777.

22. Karner, M. B., E. F. DeLong, and D. M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature. 409(6819):507-510.

23. Lebaron, P., P. Servais, H. Agogue, C. Courties, and F. Joux. 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Applied & Environmental Microbiology. 67(4):1775-1782.

24. McGinley, J. N., K. K. Knott, and H. J. Thompson. 2000. Effect of fixation and epitope retrieval on BrdU indices in mammary carcinomas. Journal of Histochemistry & Cytochemistry. 48(3):355-362.

25. Moran, R., Z. Darzykiewicz, L. Staiano-Coico, and M. R. Melamed. 1985. Detection of 5-bromodeoxyuridine (BrdUrd) incorporation by monoclonal antibodies: role of the denaturation step. Journal of Histochemistry & Cytochemistry. 33:821-827.

26. Moriarty, D. J. W. 1986. Measurement of Bacterial-Growth Rates in Aquatic Systems From Rates of Nucleic-Acid Synthesis. Advances in Microbial Ecology. 9:245-292.

27. Ouverney, C. C., and J. A. Fuhrman. 2001. Marine planktonic archaea take up amino acids. Applied and Environmental Microbiology. 67(2):1023-1023.

28. Pedros-Alio, C., and S. Y. Newell. 1989. Microautoradiographic study of thymidine uptake in brackish waters around Sapelo Island, Georgia, USA. Marine Ecology Progress Series. 55:83-94.

29. Pernthaler, A., J. Pernthaler, and R. Amann. submitted. Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria. Applied & Anvironmental Microbiology.

30. Pernthaler, A., C. M. Preston, J. Pernthaler, E. F. DeLong, and R. Amann. 2002. A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Applied and Environmental Microbiology. 68:661-667.

31. Pernthaler, J., F. O. Glöckner, W. Schönhuber, and R. Amann. 2001. Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, p. 207-226. *In* J. H. Paul (ed.), Methods In Microbiology, vol. 30. Academic Press, San Diego.

32. Petruska, J., and D. Horn. 1983. Sequence-specific responses of restriction endonucleases to bromodeoxyuridine substitution in mammalian DNA. Nucleic Acids Research. 11(8):2495-510.

33. Raap, A. K., J. G. J. Marijnen, J. Vrolijk, and M. Van der Ploeg. 1986. Denaturation, renaturation, and loss of DNA during in situ hybridization procedures. Cytometry. 7:235-242.

34. Rizzoli, R., N. M. Maraldi, A. Galanzi, N. Zini, M. Falconi, M. Vitale, and G. Mazzotti. 1988. High-Sensitivity Detection of Dna-Synthesis By Immunolocalization of Bromodeoxyuridine. Institute of Physics Conference Series(93):551-552.

35. Rodriguez, G., D. Phipps, K. Ishiguro, and H. Ridgway. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Applied and Environmental Microbiology. 58(6):1801-1808.

36. Rutter, W. J., R. C. Pictet, and M. P.W. 1973. Toward molecular mechanisms of developement processes. Annu Rev Biochem. 42:601-64.

37. Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig, and R. Amann. 1999. In situ identification of cyanobacteria with horseraddish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology. 65(3)):1259-1267.

38. Shapiro, H. M. 1995. Practical Flow Cytometry, 3rd ed. Wiley-Liss, Inc., New York.

39. Shi, S.-R., M. E. Key, and K. L. Kalra. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. Journal of Histochemistry & Cytochemistry. 39:741-748.

40. Shiah, F. K., and H. W. Ducklow. 1995. Multiscale Variability in Bacterioplankton Abundance, Production, and Specific Growth-Rate in a Temperate Salt-Marsh Tidal Creek. Limnology and Oceanography. 40(1):55-66.

41. Simek, K. 1986. Bacterial Activity in a Reservoir Determined By Autoradiography and Its Relationship to Phytoplankton and Zooplankton. Internationale Revue der Gesamten Hydrobiologie. 71(5):593-612.

42. Speel, E. J. M., F. C. S. Ramaekers, and A. H. N. Hopman. 1997. Sensitive multicolor fluorescence in situ hybridization using catalyzed reporter deposition (CARD) amplification. Journal of Histochemistry & Cytochemistry. 45(10):1439-1446.

43. Steward, G. F., and F. Azam. 1999. Bromodeoxyuridine as an alternative to H-3-thymidine for measuring bacterial productivity in aquatic samples. Aquatic Microbial Ecology. 19(1):57-66.

44. Takagi, S., M. L. McFadden, R. E. Humphreys, B. A. Woda, and T. Sairenji. 1993. Detection of 5-Bromo-2-Deoxyuridine (Brdurd) Incorporation With Monoclonal Anti-Brdurd Antibody After Deoxyribonuclease Treatment. Cytometry. 14(6):640-648.

45. Urbach, E., K. L. Vergin, and S. J. Giovannoni. 1999. Immunochemical detection and isolation of DNA from metabolically active bacteria. Applied & Environmental Microbiology. 65(3):1207-1213.

46. Van heusden, J., P. de Jong, F. Ramaekers, H. Bruwiere, M. Borgers, and G. Smets. 1997. Fluorescein-labeled tyramide strongly enhances the detection of low bromodeoxyuridine incorporation levels. Journal of Histochemistry & Cytochemistry. 45(2):315.

47. Wilson, D. M., and C. Bianchi. 1999. Improved immunodetection of nuclear antigens after sodium dodecyl sulfate treatment of formaldehyde-fixed cells. Journal of Histochemistry & Cytochemistry. 47(8):1095-1100.

48. Yin, B., D. Crowley, G. Sparovek, W. J. De Melo, and J. Borneman. 2000. Bacterial functional redundancy along a soil reclamation gradient. Applied & Environmental Microbiology. 66(10):4361-4365.

49. Zubkov, M. V., B. M. Fuchs, P. H. Burkill, and R. Amann. 2001. Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Applied and Environmental Microbiology. 67(11):5210-5218.

Publikationsliste

Glöckner FO., Zaichikov E., Belkova N., Denissova L., Pernthaler J., Pernthaler A., Amann R. (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. *Applied & Environmental Microbiology*. **66**(11):5053-65.

Pernthaler J., Posch T., Simek K., Vrba J., Pernthaler A., Glöckner FO., Nübel U., Psenner R., Amann R. (2001). Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. *Applied & Environmental Microbiology*. **67**(5):2145-55.

Dubilier N., Mülders C., Ferdelman T., de Beer D., Pernthaler A., Klein M., Wagner M., Erseus C., Thiermann F., Krieger J., Giere O., Amann R. (2001). Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature*. **411**(6835):298-302.

Pernthaler A., Pernthaler J., Eilers H., Amann R. (2001). Growth patterns of two marine isolates: Adaptations to substrate patchiness? *Applied & Environmental Microbiology*. **67**(9):4077-4083.

Swidsinski A., Ladhoff A., Pernthaler A., Swidsinski S., Loening-Baucke V., Ortner M., Weber J., Hoffmann H., Dietel M., Lochs H. (2002). Impaired mucosal clearance of intraluminal bacteria in inflammatory bowel disease described with bacterial culture, quantitative PCR, sequencing, electron microscopy and fluorescence in-situ hybridization. *Gastroenterology*. **122**:44-54.

Pernthaler A., Preston C.M., Pernthaler J., DeLong E.F., Amann R. (2002). A comparison of fluorescently labeled oligonukleotide and polynukleotide probes for the detection of pelagic marine bacteria and archaea. *Applied & Environmental Microbiology*. **68**(2):661-667.

Pernthaler A., Pernthaler J., Amann R. (in press). Fluorescence in situ hybridization and catalyzed reporter deposition (CARD) for the identification of marine bacteria. *Applied & Environmental Microbiology*.

Pernthaler A., Pernthaler J., Schattenhofer M., Amann R. (submitted). Detection and identification of DNA-synthesizing bacteria in coastal North Sea plankton. *Applied & Environmental Microbiology*

DANKSAGUNG

Der Betreuer (unter anderem):
Jakob Pernthaler *(Ohne Dich wäre das Leben nicht mal halb so schön! Danke für Deine Liebe,Deinen Enthusiasmus, und die vielen Abende mit Rotwein und Forschung! Nachtrag: ...für Rettung in letzter Minute (Sonntachnacht am Computer)*

Die Familie:
Karin Hentschke *(Die es nie geschafft hat, mir Ordungsliebe beizubringen)*
Jenny Hentschke *(Die es letztendlich doch geschafft hat, mir „einen festen Freund" einzureden)*
Otti Link *(Die mit Weisheit, Wärme und Buchteln ihre Enkel verwöhnt)*
Alois Link *(Der uns immer zum Lachen gebracht hat)*
Lars Hentschke *(„Die Annelie war's!")*
Klaus Hentschke *(Der Mann mit dem größten Mikroskops Ostdeutschlands)*

Der Chef:
Rudolf Amann *(...wenn ich mal groß bin, werd ich so wie Du)*

Die Bürofamilie:
Bernhard „Barney" Fuchs *(Gut zielen lernen mit Barney: wie man die Kollegen mit fliegenden Pritt-Stiften u.a. harten Gegenständen bei Laune halten kann)*
Sebastian „Waschtl" Behrens *(Der Wolf im Schafspelz! Ohne Scheiss!)*
Falk Warnecke *(Der einzige Mann der gern High Heels anzieht und dabei auch noch gut aussieht)*
Torben „Törbsche" Stührman *(Der leider nie für mich arbeiten wollte und warscheinlich auch gut aussieht in High Heels)*

Kollegen, Exkollegen und vielleicht zukünftige Kollegen:
Uli Nübel *(The sexiest man alive! - logisch)*
Mark Mußmann *(Der herausfand, daß man Alkohol auch toll inhalieren kann)*
Enrique „Quique" Llobett Brossa *(Pantalones! ...das andere ist Schweinkram)*
Barbara McGregor *(The one & only „brownie & cranberrie sauce & muffins – maker")*
Ramon Rossellio Mora *(Hat mich im „Va Bene" zur genialen Forscherin gemacht)*
Nicole Dubilier *(Die rabiat Ehrliche, und das ist auch gut so!)*
Christina Preston *(Science fiction with cocktails & whirl pool)*
Jörg Wulf *(„das Protokoll hab' ich mal modifiziert")*
...und den Rest der Mollies *(danke für die lustigen Gruppenfrühstücke, Julefrokoste oder Julefrokosts, late-summer-special-Kalorienbomben und alles andere, was dick macht und von der Arbeit abhält)*

Die auch unter mir leiden mußten:
Martin Skuppe *(Wir haben die geilsten Fische!)*
Wolfgang Tlok *(„ein Bier, ein Korn")*