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Phylotype-specific growth rates of marine bacteria measured by bromodeoxyuridine immunocytochemistry and fluorescence *in situ* hybridization

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ABSTRACT: To explore the possibility of calculating phylotype-specific growth rates of marine bacteria, we optimized a protocol that combines bromodeoxyuridine (BrdU) immunocytochemistry with fluorescence *in situ* hybridization (FISH). The protocol worked well with 8 isolates tested, showing a significant positive correlation between average cellular fluorescence intensity and cell-specific BrdU contents. The BrdU immunocytochemistry-FISH (BIC-FISH) was then applied to neritic seawater in Hiroshima Bay, western Japan. *Rhodobacter/Roseobacter* and *Bacteroidetes* groups were dominant in BrdU-positive cells (22 ± 8 and 26 ± 8%), suggesting a significant contribution to bacterial community productivity in the water. Analysis of single-cell immunofluorescence showed higher BrdU incorporation rates in the *Gammaproteobacteria*, SAR86, and *Vibrio* groups, suggesting that these less abundant groups were growing more rapidly than other phylotypes. Our study suggests that BIC-FISH has the potential to estimate phylotype-specific variability of bacterial productivity in aquatic systems.

KEY WORDS: Bromodeoxyuridine · Immunocytochemistry · FISH · Growth rate

INTRODUCTION

Bromodeoxyuridine (BrdU), a halogenated nucleoside, serves as a thymidine (TdR) analog and has been recently used (Hamasaki et al. 2007, Taniguchi & Hamasaki 2008) as a tracer of de novo DNA synthesis in marine bacterial assemblages. The BrdU incorporation immunoassay has been successfully used to measure secondary production in natural bacterial assemblages as an alternative to the [^{3}H]-TdR incorporation radioassay (Steward & Azam 1999, Nelson & Carlson 2005, Hamasaki 2006). BrdU-incorporating bacteria can be visualized at the single-cell level by using anti-BrdU antibodies conjugated with fluorochrome molecules or enzymatic reporter molecules (Urbach et al. 1999, Hamasaki et al. 2004). This single-cell-based method has been combined with 16S rRNA fluorescence *in situ* hybridization (FISH) to detect DNA-synthesizing bacteria and determine their phylogenetic subgroups (Pernthaler et al. 2002). The percentages of BrdU-positive cells can be compared among subgroups of bacteria as an indicator of phylotype-specific population growth rates.

The procedure combining BrdU immunocytochemistry and FISH (BIC-FISH) requires special precautions to effectively detect the fluorescence signals from both assays. In previous studies, filters were coated with low-gelling-point agarose after sample filtration to prevent cell detachment during treatment steps (Pernthaler et al. 2002, Pernthaler & Pernthaler 2005). However, the agarose matrix layer on the filter can limit access by chemicals and may lead to reductions in staining efficiency and detection sensitivity. To develop a more effective and sensitive method, we tested poly-L-lysine (PLL)-coated filters. These filters are effective in retaining cells during FISH treatment (Maruyama & Sunamura 2000); the polycationic nature of PLL allows interaction with the anionic sites of bacterial cell walls, resulting in strong adhesion.

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We also tested an advanced application of BIC-FISH to calculate phylotype-specific growth rates at the single-cell level by measuring immunofluorescence intensity. Microautoradiography has been recently combined with FISH to assess phylotype-specific substrate uptake at the single-cell level (Lee et al. 1999), and tritiated thymidine can be used as a substrate for indicating the growth of cells (Fuhrman & Azam 1982). However, microautoradiography-FISH requires the use of radioisotopes and involves the cumbersome quantification of silver grains. Previous results from the single-cell-based BrdU method showed a significant relationship between BrdU immunofluorescence intensity and bacterial growth rates (Hamasaki et al. 2004). Here we explored the possibility of calculating cellular BrdU incorporation rates (presumably reflecting single-cell growth rates) from immunofluorescence intensity.

Our specific goals were (1) to optimize the BIC-FISH methodology, (2) to evaluate the relationship between cellular BrdU content and immunofluorescence intensity, and (3) to apply the method to seawater bacterial assemblages to compare phylotype-specific growth rates and determine the relative contribution of each phylotype to total bacterial productivity.

MATERIALS AND METHODS

BrdU immunocytochemistry (BIC)-FISH. Samples for BIC-FISH analysis (seawater or cultured cell suspension) were filtered through PLL-coated membrane filters (see next section) to collect bacterial cells. The membrane filters were cut into small pieces (up to 12) and then dehydrated by serial treatment with 70, 90, and 100% ethanol, each for 3 min. To quench endogeneous peroxidase activity in the samples, the filters were treated by soaking 3% H_2O_2 in phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 4.3 mM $NaH_2PO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4 [pH 7.0]) for 10 min at room temperature, and then washed with 1 ml PBS for 10 min. Filters were then processed by soaking in 0.01 M HCl for 5 min at room temperature. They were then soaked in pepsin (0.5 mg ml^{-1} in 0.01 M HCl; Sigma-Aldrich) for 2 h at 37°C, washed with 1 ml PBS for 10 min, treated with lysozyme (10 mg ml^{-1} in 100 mM Tris HCl and 50 mM EDTA [pH 8.0]) for 15 min at room temperature, and washed with 1 ml PBS for 10 min. Intracellular DNA was denatured by treatment with nucleases (diluted 1:100 in the incubation buffer included with the BrdU Labeling and Detection Kit III; Boehringer) for 2 h at 37°C. This was followed by a wash with 1 ml PBS for 10 min.

The filters were placed on a glass slide with the cell-adherent side facing down in contact with a 30 µl drop of the hybridization solution containing 2.5 ng $µl^{-1}$ of biotin-labeled FISH probes. The probes used are listed in Table 1. To distinguish single nucleotide differences between Bet42a and Gam42a probes, unlabeled oligonucleotide Gam42a or Bet42a was mixed into the hybridization solutions as a competitor probe (Manz et al. 1992). Hybridization continued for 180 min at 42°C, after which the filters were washed with 1 ml washing buffer for 15 min at 48°C. The FISH signal was amplified by tyramide signal amplification (TSA). The filters were incubated with HRP-labeled streptavidin diluted 1:50 in TNB buffer (100 mM Tris HCl [pH 7.5], 150 mM NaCl, and 0.5% w/v blocking reagent from the TSA-Indirect kit [Perkin Elmer Life Sciences]) for 30 min at room temperature and then washed with 1 ml TNT buffer (100 mM Tris HCl [pH 7.5], 150 mM NaCl, and 0.05% v/v Tween 20) for 10 min. After the filters were washed with TNT buffer, they were treated with a biotin-labeled tyramide and then Texas Red-labeled streptavidin in TNB buffer (1:500) for 30 min at room temperature and washed with 1 ml TNT buffer for 10 min. To quench peroxidase in the samples, the filters were treated with 0.1 M HCl for 10 min at room temperature and then washed with 1 ml PBS for 10 min.

After permeabilization, DNA denaturation, and TSA-FISH, the samples were treated for 120 min at 37°C with anti-BrdU monoclonal antibodies conjugated with peroxidase diluted in TNB buffer (1:50) and

Table 1. Oligonucleotide sequences of FISH probes used in this study. The target site is based on numbering of base pairs in *Escherichia coli*

Probe	Specificity	Probe sequence (5′–3′)	Target site	Source
Eub338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S (338–355)	Amann et al. (1990)
Non338	Negative control	ACTCCTACGGGAGGCAGC	16S (338–355)	Amann et al. (1990)
Alf968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	16S (968–986)	Glöckner et al. (1999)
Bet42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	23S (1027–1043)	Manz et al. (1992)
Gam42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	23S (1027–1043)	Manz et al. (1996)
Cf319a	*Bacteroidetes* group	TGGTCCGTGTCTCAGTAC	16S (319–336)	Manz et al. (1996)
G Rb	*Roseobacter/Rhodobacter* group	GTCAGTATCGAGCCAGTGAG	16S (626–645)	Giuliano et al. (1999)
G V	*Vibrio* group	AGGCCACAACCTCCAAGTAG	16S (841–822)	Giuliano et al. (1999)
SAR86-1249	SAR86 group	GGCTTAGCGTCCGTCTG	16S (1249–1265)	Eilers et al. (2000)

then washed with 1 ml PBS. The antibody signal was amplified by incubating the filters with biotin-labeled tyramide diluted 1:50 in amplification buffer (included with the BrdU Labeling and Detection Kit III) for 10 min at room temperature. The filters were washed with 1 ml TNT buffer for 10 min, treated with fluorescein isothiocyanate (FITC)-labeled streptavidin in TNB buffer (1:500) for 30 min at room temperature, and then washed again with TNT buffer. The samples were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 µg ml^{-1}) for 5 min and washed with PBS.

The filters were placed on glass slides with anti-fading solution (1:4 mixture of Vectashield [Vector Labs] and Citifluor [Citifluor]) and cover glasses. The slides were examined under an Olympus BX51 epifluorescence microscope (Olympus Optical) equipped with a CoolSNAP CCD camera (Roper Scientific).

Comparison of cell-adherent solutions and permeabilization conditions. For this comparison, a seawater sample was collected from Hiroshima Bay, Japan, in April 2005 and incubated with 1 µM BrdU at *in situ* temperature for 5 h to label the DNA of bacteria. The samples were filtered onto PLL-coated membrane filters, and also onto uncoated filters that were coated with agarose after the sample filtration (see below). After BrdU immunocytochemistry treatment of the filters using 3 different antibody concentrations, the number of cells retained and the percentage of immunofluorescence-positive cells on both filter types were counted (Table 2). PLL-coated filters were prepared by dipping 0.22 µm pore size polycarbonate membrane filters (25 mm, type GTTP; Millipore) in a PLL solution (0.01% [w/v] in distilled water; Sigma-Ardrich; Maruyama & Sunamura 2000). Filters were dried and stored at room temperature in a Petri dish until used for filtration. Filters were coated with agarose after sample filtration; 0.22 µm pore size polycarbonate membrane filters containing bacterial cells were dipped in agarose solution (0.2% low-melting-temperature agarose [Nacalai Tesque]) and dried on glass slides at 46°C (Pernthaler et al. 2002).

Table 2. Number of cells retained on filters after bromodeoxyuridine (BrdU) immunocytochemical treatment, and percentage of BrdU-positive cells detected following BIC-FISH on PLL- and agarose-coated filters using different concentrations of anti-BrdU antibody. Data are means ± 1 SD

Dilution anti-BrdU antibody	Retained cells (×10^6 cells ml^{-1})		Percentage of BrdU-positive cells (% of DAPI-stained cells)	
	PLL-coated	Agarose-coated	PLL-coated	Agarose-coated
1:500	3.8 ± 0.2	3.9 ± 0.2	2.6 ± 1.4	2.3 ± 1.0
1:100	3.9 ± 0.2	3.4 ± 0.2	13.5 ± 1.7	5.9 ± 2.0
1:50	3.8 ± 0.2	3.8 ± 0.2	26.0 ± 4.8	7.7 ± 2.3

Table 3. Fluorescence intensities of FISH signals after permeabilization using different pepsin concentrations and incubation times. Signal intensity is indicated by the number of plus symbols. –: not detected; empty cells: no data

Pepsin concentration (mg ml^{-1})	Incubation time (min)		
	0	60	120
0	–		
0.2	–	–	+
0.5	–	–	++
1.0	–	–	+
2.0		–	–

To optimize permeabilization, the samples filtered onto PLL-coated filters were processed for BIC-FISH by the procedures already described but with modified pepsin concentrations and incubation times (Table 3). Excessive permeabilization can cause loss of rRNA from the cells. Thus, the optimization of this treatment step was the most sensitive for successfully combining BrdU immunocytochemistry with FISH. In our protocol, the cell wall and membrane were permeabilized by using pepsin and lysozyme. We examined the immunofluorescence intensity for each set of permeabilization conditions. All data are mean ± SD.

Isolation and phylogenetic identification of marine bacteria. Marine bacteria were isolated from surface seawater at Sagami Bay (35° 09′ N, 139° 10′ E), central Japan, by using a ZoBell 2216E agar plate. The 16S rRNA gene was amplified with bacterial universal primers (Lane 1991). Ex-*Taq* (TaKaRa) polymerase was used to amplify the genes. The PCR products were purified with an EXOSAP-IT kit (USB), sequenced using an ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems), and an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequences were aligned with known sequences in the DNA Data Bank of Japan (DDBJ) using the Basic Local Alignment Search Tool (BLAST).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the isolates (M12, M16 etc.) have been assigned the following GenBank accession numbers: isolate M12, *Thalassospira* sp., AB520756; M16, *Erythrobacter* sp., AB520757; M17, *Erythrobacter* sp., AB520758; M20, *Erythrobacter* sp., AB520759; M3, *Photobacterium* sp., AB520760; M14, *Stenotrophomonas* sp., AB520761; M22, *Stenotrophomonas* sp., AB520762; M19, *Tenacibaculum* sp., AB520763.

Cellular BrdU content and immunofluorescence intensity. The isolates were cultured in SWM medium (GF/F filtered seawater containing 0.0058%

[w/v] casamino acids, 0.69 mM glucose, 0.30 mM NH_4Cl, 0.25 mM NaH_2PO_4, 0.2 µM ferric citrate, 0.2 µM EDTA, and 0.001% [w/v] BACTO peptone [BD Diagnostic]) until they reached exponential growth phase. They were then labeled with 200 nM BrdU at 23°C. Isolates M3, M14, and M19 were subsampled at 0, 0.5, 1, 2, and 3 h, and isolates M12, M16, M17, M20, and M22 were subsampled 0, 0.5, 2, and 3 h after the addition of BrdU. An aliquot of each subsample was immediately fixed with 2% paraformaldehyde and kept at 4°C overnight. The fixed samples were filtered onto PLL-coated filters and subjected to BIC-FISH treatment. Another aliquot of the subsample was supplemented with excess TdR (100 µM final concentration) to stop further incorporation of BrdU and kept at 4°C until all subsampling was completed. These subsamples were filtered onto a 0.2 µm pore size nylon membrane (MSI) by using a dot blotter (Minifold; Schleicher and Schuell) and used for a chemiluminescence immunoassay of BrdU against extracted DNA fractions (Steward & Azam 1999, Hamasaki 2006). The nylon membranes were treated with solutions for lysing bacterial cells, exposed to UV light to crosslink DNA, and further processed to measure the total amount of BrdU incorporated into bacterial cells according to a previously described method (Hamasaki 2006). Cellular BrdU contents were calculated from the total amount of BrdU and the number of BrdU-positive cells. In addition, cell-specific immunofluorescence intensity was represented as the average intensity of 30 to 50 individual cells in microscopic images obtained from BIC-FISH (see 'Image analysis' section for details).

Application to seawater bacterial assemblages. The optimized BIC-FISH procedure was used to assess the phylotype-specific growth characteristics (percentages of BrdU-positive cells and cellular BrdU incorporation rates) of major bacterial subgroups in seawater assemblages. A seawater sample was collected from 5 m depth at a pier in Kure Port (34° 14′ N, 132° 33′ E) in Hiroshima Bay, western Japan, in October 2005. The seawater was screened through a 200 µm pore size nylon mesh to remove zooplankton. Aliquots of the seawater were incubated with 1 µM BrdU for 3 h in dark bottles at the *in situ* temperature ±2°C and fixed with 2% parafolmaldehyde for 24 h at 4°C. Negative controls for the BrdU immunoassay were obtained from subsamples collected before the incubation with BrdU. The fixed samples were filtered onto PLL-coated 0.2 µm pore size polycarbonate white membrane filters, washed twice with distilled water, and then stored at −80°C until BIC-FISH treatment.

Image analysis. Epifluorescence microscope images captured by a cooled CCD camera were analyzed with the image-analysis software WinROOF (MitaniCorp) to enumerate bacterial cells and with Image-Pro Plus 5.1J (Media Cybernetics) to measure fluorescence intensity. For counting cells made visible by BIC and FISH, the threshold for visualization in the images was first set to the gray value represented by the cells in negative controls, and then the images were processed using constriction and dilatation tools to remove noise particles. Three images (BIC, FISH, and DAPI) captured from 1 field were individually processed, and 2 or 3 out of the 3 processed images were combined into 1 image with the 'AND' function. The resulting images were processed for counting from 300 to 750 cells sample^{-1}. Also, 30 to 50 cells in each sample were analyzed at random to measure the immunofluorescence intensity of BrdU-positive cells.

RESULTS

BIC-FISH

Observations with fluorescence microscopy showed that we achieved successful triple staining with DAPI, BrdU immunofluorescence, and FISH for all bacterial strains and natural samples that we tested. Fig. 1 shows examples of the photomicrographs obtained. BrdU immunofluorescence signals from FITC were obtained from samples incubated for 2 h (Fig. 1g,h), with no signals in the 0 h sample (Fig. 1e,f). The Eub338 probe produced FISH signals of Texas Red (Fig. 1i,k), with no signals visible from the Non338 probe as a negative control (Fig. 1j,l).

Comparison of cell-adherent treatments and permeabilization conditions

There was no difference between PLL-coated and agarose-coated filters in the number of cells retained on the filter (Table 2). However, PLL-coated filters had a higher percentage of BrdU-positive cells than agarose-coated filters when the antibody concentration was increased. At a 1:50 anti-BrdU antibody dilution, the percentages of BrdU-positive cells were 26 ± 5% for PLL-coated filters and 7.7 ± 2.3% for agarose-coated filters. The best results in the permeabilization comparison were achieved by applying 0.5 mg ml^{-1} pepsin for 120 min with a 1:50 antibody dilution (Table 3).

Cellular BrdU content and immunofluorescence intensity

Average cellular BrdU contents were calculated from the bulk measurement of BrdU incorporation and

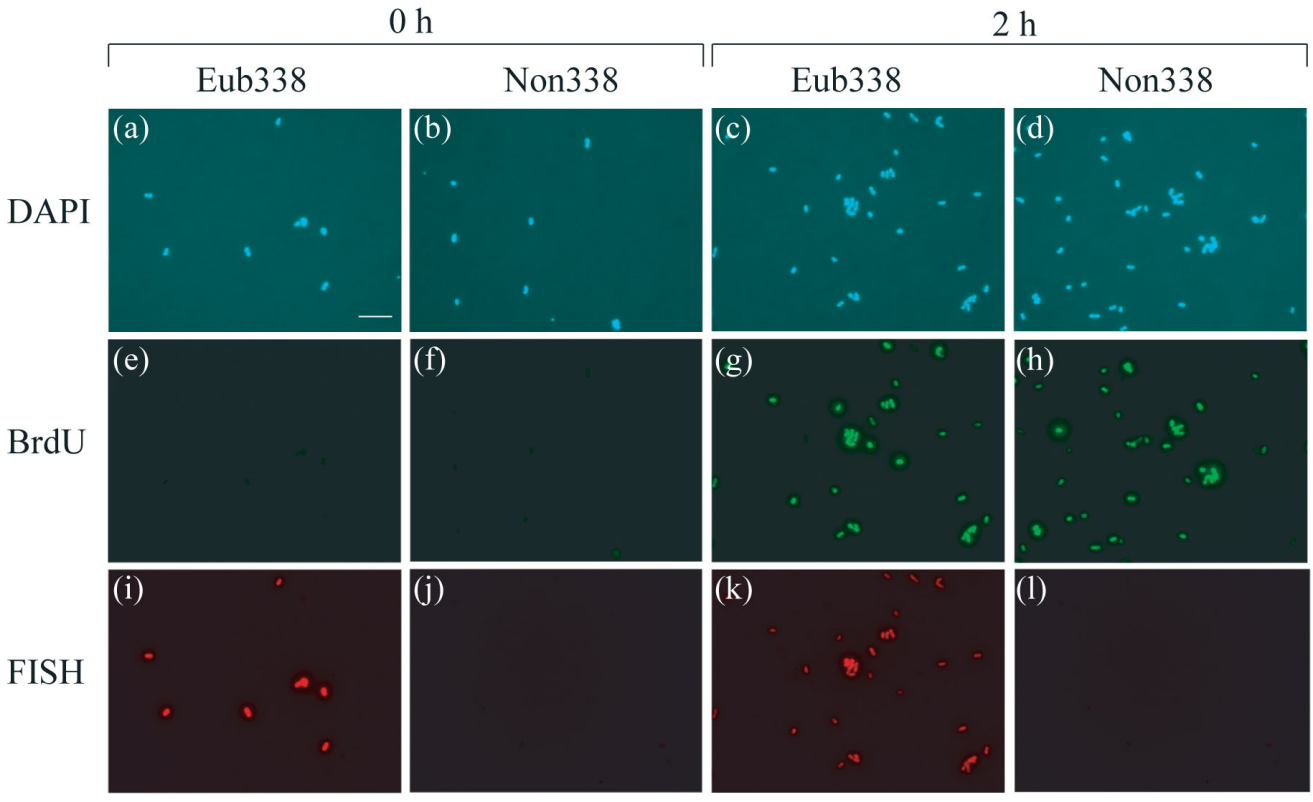

Fig. 1. Representative photomicrographs showing the fluorescence of bacterial cells (isolated *Gammaproteobacteria* strain M3; *Photobacterium* sp.) stained with DAPI (a–d), with bromodeoxyuridine (BrdU) immunocytochemistry-fluorescence *in situ* hybridization (BIC-FISH; e–h), and with FISH probes (Texas Reds Eub338 [i, k] and Non338 [as a negative control; j, l]). After collecting the negative control samples (0 h), bacteria were labeled with BrdU for 2 h. BrdU signals from fluorescein isothiocyanate are visible in the 2 h sample (g, h) but not in the 0 h sample (e, f). Micrographs c, g, and k represent successful triple staining (DAPI, BrdU, and FISH). Scale bar: 10 µm

the number of BrdU-positive cells, as counted by immunocytochemistry. The cellular BrdU contents increased during the log growth phase of incubation. The average immunofluorescence intensity of all isolates showed significant correlations with average cellular BrdU contents (M12: $r^2 = 0.947$, $n = 4$, $p < 0.05$; M16: $r^2 = 0.997$, $n = 4$, $p < 0.01$; M17: $r^2 = 0.995$, $n = 4$, $p < 0.01$; M20: $r^2 = 0.984$, $n = 4$, $p < 0.01$; M3: $r^2 = 0.944$, $n = 5$, $p < 0.01$; M14: $r^2 = 0.847$, $n = 5$, $p < 0.05$; M22: $r^2 = 0.953$, $n = 4$, $p < 0.05$; M19: $r^2 = 0.910$, $n = 5$, $p < 0.05$; Fig. 2). There were no significant differences between the isolates for the slopes of the regression lines describing the relationship between BrdU fluorescence intensity and content per cell (analysis of variance [ANOVA], $p > 0.05$).

Application to seawater bacterial assemblages

The Eub338 probe detected 60 ± 8% of the DAPI-stained bacteria. The *Bacteroidetes* group was predominant (ANOVA, Tukey-Kramer post hoc test, $p < 0.05$) and accounted for 16 ± 6% of the DAPI-stained community (Fig. 3). The SAR86 group affiliated with *Gammaproteobacteria* accounted for 10 ± 3%, *Alphaproteobacteria* accounted for 8 ± 4%, and the *Rhodobacter/Roseobacter* group within *Alphaproteobacteria* represented 7 ± 2% of the DAPI-stained community. The *Betaproteobacteria* comprised <1% of the DAPI-stained community.

BrdU-positive cells accounted for about 19 ± 3% of DAPI-stained cells. Bacterial cells detected by the Eub338 probe accounted for 81 ± 3% of the total BrdU-positive community. *Bacteroidetes* and *Rhodobacter/Roseobacter* were the 2 dominant groups in the BrdU-positive community (ANOVA, Tukey-Kramer post hoc test, $p < 0.05$) and accounted for 26 ± 8%, and 22 ± 8% of the BrdU-positive cells, respectively (Fig. 3). The *Betaproteobacteria* were <1% of the BrdU-positive community. The *Gammaproteobacteria* were 6 ± 7% of the BrdU-positive community.

We also calculated the percentage of FISH-positive cells in each group that were BrdU-positive (Fig. 4). BrdU-positive cells accounted for 27 ± 7% of Eub338-positive cells. BrdU-positive cells were relatively abundant in the *Alphaproteobacteria*, *Betaproteobacteria*,

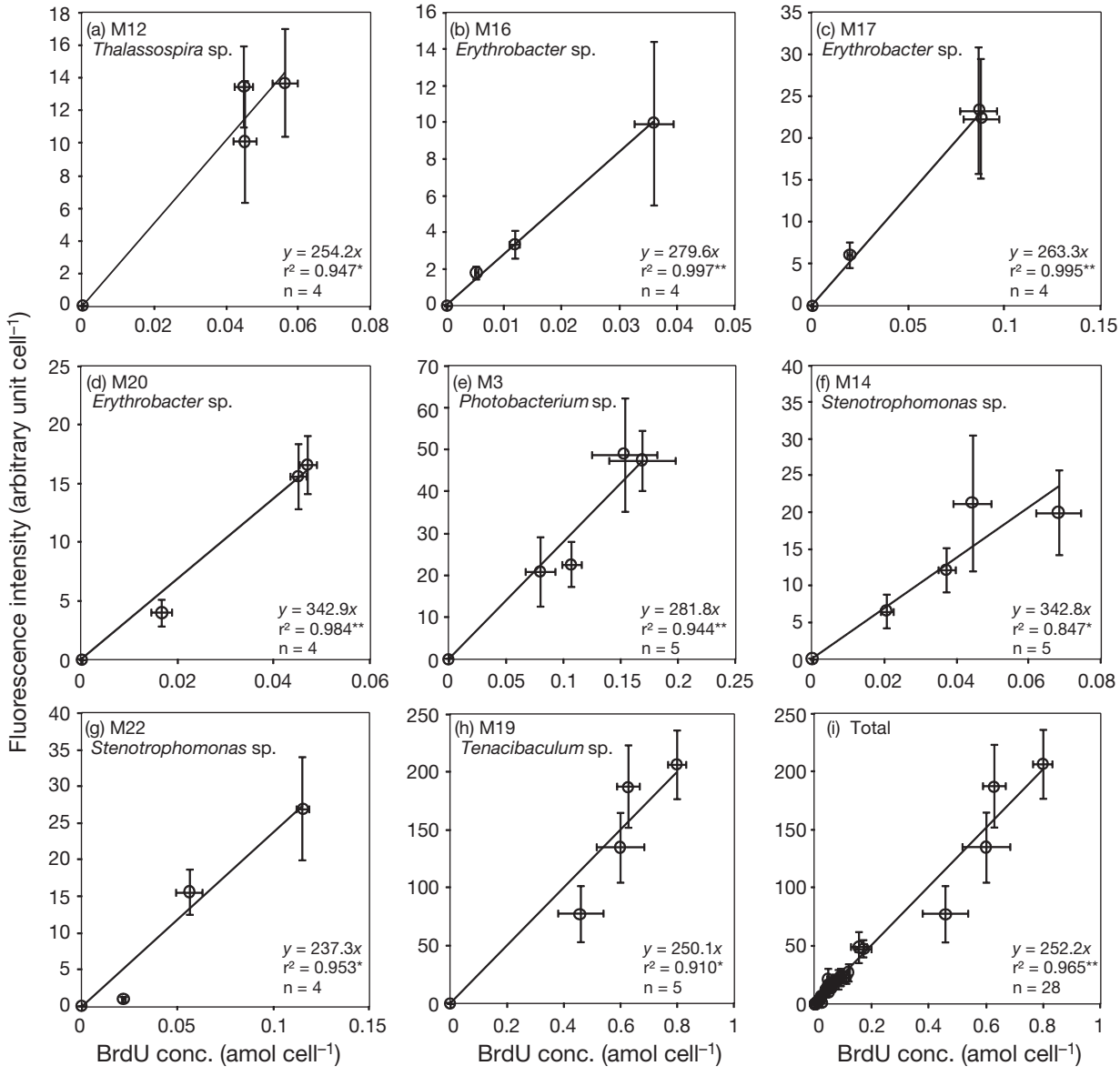

Fig. 2. Relationships between cellular bromodeoxyuridine (BrdU) content and average cellular fluorescence intensity. Isolates were labeled with 200 nM BrdU (see 'Materials and methods'). (a–d) *Alphaproteobacteria* isolates *Thalassospira* sp. (a) and *Erythrobacter* sp. (b–d). (e–g) *Gammaproteobacteria* isolates *Photobacterium* sp. (e) and *Stenotrophomonas* sp. (f, g). (h) *Bacteroidetes* isolate (*Tenacibaculum* sp.). (i) Data from all isolates combined. Subsamples from each isolate were subjected to bulk measurement of incorporated BrdU and also to immunocytochemistry to permit counting of BrdU-positive cells and to calculate cellular BrdU content. Error bars indicate ±1 SD. $^{*}p < 0.05$; $^{**}p < 0.01$

Rhodobacter/*Roseobacter*, and *Vibrio* groups, accounting for 50 ± 11, 53 ± 25, 56 ± 9, and 47 ± 19%, respectively. The proportions of BrdU-positive cells in the *Betaproteobacteria* and *Vibrio* group were variable, with coefficients of variation (CVs) of 48 and 40%, respectively. In contrast, the proportions of BrdU-positive cells in the *Gammaproteobacteria*, *Bacteroidetes*, and SAR86 groups were relatively low and accounted for 40 ± 18, 36 ± 7, and 28 ± 14%, respectively. The numbers of BrdU-positive cells in *Gammaproteobacteria* and the SAR86 group were variable, with CVs of 44 and 50%, respectively.

We measured the immunofluorescence intensity of individual cells (Fig. 5), differentiating specific phylotypes. The average fluorescence intensity of the *Gammaproteobacteria* was significantly higher than those of other phylotypes, except for the *Betaproteobacteria*, SAR86, and *Vibrio* groups (ANOVA,

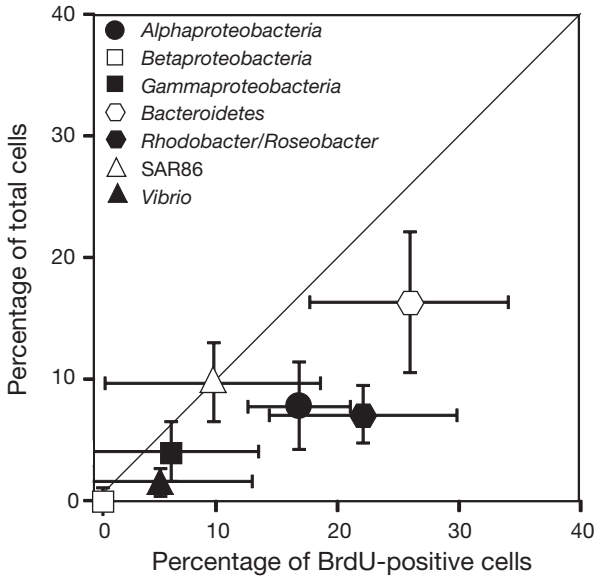

Fig. 3. Relationship between percentage of each phylogenetic group in total bacterial cells and percentage in BrdU-positive cells. The seawater sample was collected from a pier in Kure Port in Hiroshima Bay, western Japan, in October 2005. The sample was labeled with 1 µM BrdU for 3 h and subjected to BIC-FISH analysis. Error bars indicate ±1 SD

Tukey-Kramer post hoc test, $p < 0.001$). The intensities in the *Bacteroidetes* and *Rhodobacter/Roseobacter*, which dominated the BrdU-positive cells, were lower than those of the other phylotypes, except for the *Alpha*- and *Betaproteobacteria* (ANOVA, Tukey-Kramer post hoc test, $p < 0.05$).

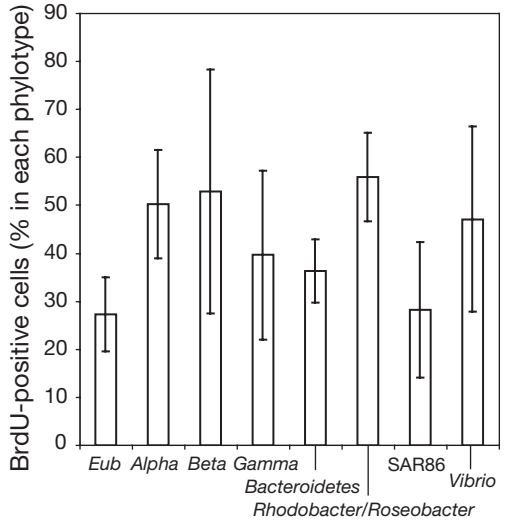

Fig. 4. Percentage of BrdU-positive cells in each phylogenetic group in a seawater sample collected from a pier in Kure Port in Hiroshima Bay, western Japan, in October 2005. The sample was labeled with 1 µM BrdU for 3 h and subjected to BIC-FISH analysis. Error bars indicate ±1 SD (ANOVA, $p < 0.001$). *Eub*: *Eubacteria*; *Alpha*: *Alphaproteobacteria*; *Beta*: *Betaproteobacteria*; *Gamma*: *Gammaproteobacteria*

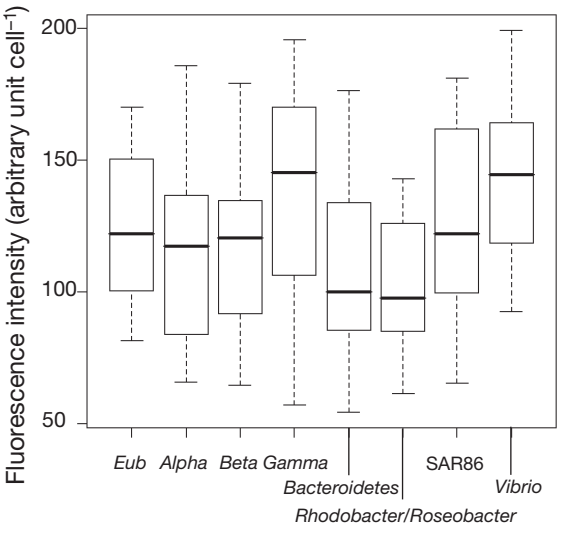

Fig. 5. BrdU immunofluorescence intensity of major phylogenetic groups of marine bacteria in a sample collected from a pier in Kure Port in Hiroshima Bay, western Japan, in October 2005. The box plot for each group indicates the smallest non-outlier observation, lower quartile, median, upper quartile, and largest non-outlier observation (ANOVA, $p < 0.001$). See Fig. 4 for phylogenetic group abbreviations

DISCUSSION

A potential limitation of techniques using BrdU is that not all microorganisms can incorporate or take up BrdU. In a study by Hewitt et al. (1967), *Escherichia coli* and *Bacillus subtilis* strains did not incorporate exogenously supplied BrdU unless they had mutations affecting thymidine synthesis. However, recent studies have shown that many wild-type bacteria isolated from lake water and seawater (in total, 61 out of 66 bacterial isolates) can incorporate BrdU (Urbach et al. 1999, Pernthaler et al. 2002, Hamasaki et al. 2004, 2007). These results suggest that BrdU incorporation methods have the potential to be broadly applicable to all major phylotypes of bacteria in pelagic marine assemblages.

In this study, we developed an optimized procedure for BIC-FISH. The method is based on the procedure of simple BrdU immunocytochemistry (Hamasaki et al. 2004) but includes several modifications and significant improvements. One improvement is that all steps of the processes can be performed while the cells remain on a membrane filter. The original procedure was performed on bacterial cells transferred to a glass slide after being collected on a membrane filter. This technique provided lower fluorescence background and higher sensitivity of detection than the procedure performed directly on the filter. However, in consideration of possible inconsistencies in the efficiency of transferring the cells from a filter to a glass slide, we recommend performing all treatment steps directly on the filter.

The most critical concern of the filter method is a possible loss of cells during the numerous treatment steps. We have overcome this problem by using filters coated with a cell-adhesive substrate, PLL. This coating was even more effective than the agarose coating used in previous studies (Pernthaler et al. 2002; our Table 2). The lower detection efficiencies obtained by using agarose-coated filters might be attributable to inefficient access of antibody molecules to their targets in the agarose-matrix layer. In this study, all major treatment steps, including permeabilization, denaturation, and antibody application, were carefully optimized specifically for BIC-FISH to yield maximum fluorescence signals (Table 3).

The step most critical to the successful optimization of the procedure was the permeabilization step. The concentration of pepsin previously optimized for simple immunocytochemistry did not work well for the procedure in combination with FISH. This pepsin concentration quenched FISH signals, probably through degradation of rRNA protein and subsequent outflow of the rRNA. We found that a reduced concentration of pepsin with a longer incubation time than in the simple BrdU procedure worked well for the immunocytochemistry combined with FISH.

The protocol for our BIC-FISH is different in some treatment steps from the one developed by Pernthaler et al. (2002). We have no data for direct comparisons between these 2 protocols. However, the percentages of BrdU-positive cells that we detected were comparable to, and sometimes higher than, those obtained in the previous study. Pernthaler et al. (2002) used only lysozyme for membrane permeabilization, but we added pepsin before the lysozyme treatment for more effective permeabilization. We adopted enzymatic digestion using nucleases to denature double-stranded DNA. Several other methods have been used for DNA denaturation in the immunochemical detection of incorporated BrdU, such as heating (Moran et al. 1985), acid treatment (Mazzotti et al. 1990, Lewis & Errington 1997), alkaline treatment (Anderson & Young 1985), and formamide treatment (Raap et al. 1986). Renaturing of once-denatured DNA strands has been identified as a problem (Raap et al. 1986). Steward & Azam (1999) tested 4 denaturing procedures — heat, nuclease, acid, and alkali — for BrdU-labeled DNA samples blotted on nylon membranes. They found that denaturation by nucleases resulted in strong signals compared to other treatments. Exonuclease (III) could increase BrdU detection sensitivity compared to treatments combining formamide, hot hydrochloric acid, and *Hae* III enzymes (Pernthaler & Pernthaler 2005). Although our higher percentage of BrdU-positive cells compared to previous reports could have been due to differences in microbial communities in the areas studied, our method is sensitive enough to determine the phylotypes of a substantial portion of the actively growing bacteria.

We explored the possibility of determining phylotype-specific growth rates of the major subgroups of marine bacteria. The cell-specific immunofluorescence intensities of isolates affiliated with *Alpha-* and *Gammaproteobacteria* and the *Bacteroidetes* groups were significantly correlated with cellular BrdU content, and the data from these groups could be combined into 1 regression line ($r = 0.98$, $n = 28$, $p < 0.001$; Fig. 2i), suggesting that the amount of BrdU incorporated into individual cells can be estimated from the immunofluorescence intensity. To convert BrdU incorporation rates into growth rates, it may be necessary to consider the variability of genome size and number of copies and the guanine/cytosine content of the phylotypes, although results from using the tritiated thymidine method have shown a significant relationship between thymidine incorporation and growth rates (Fuhrman & Azam 1982). Also, a strong relationship between thymidine and BrdU incorporation rates has already been shown (Steward & Azam 1999). Although in theory there should be some variation in the relationship between TdR incorporation and growth rate, the method has been widely applied to natural samples through the application of empirical conversion factors. The growth rates of individual cells and phylotypes could also be determined if appropriate conversion factors were available. Further study is required to determine the appropriate conversion factors.

Single-cell-based analysis of natural seawater bacterial assemblages showed that the *Rhodobacter/ Roseobacter* and *Bacteroidetes* groups accounted for relatively high percentages of the total and BrdU-positive bacteria (Fig. 3), although these groups had relatively low fluorescence intensities (with the exception of the *Alpha-* and *Betaproteobacteria*; ANOVA, Tukey-Kramer post hoc test, $p < 0.05$; Fig. 5). Studies using microautoradiography-FISH have revealed that the *Roseobacter* group always dominated the substrate uptake fractions and seemed to adjust to different trophic conditions, whereas the percentages of *Bacteroidetes* increased at higher substrate levels (Alonso & Pernthaler 2006, Alonso-Sáez & Gasol 2007). Our data suggest that the contributions of these groups to the total bacterial production in our samples were determined by their population size rather than by their single-cell activities. Phylotype detection using FISH identifies fairly broad ranges of prokaryotes, thus including a number of species possessing various growth characteristics and in various growth stages. This can cause large variability in the BrdU immunofluorescence intensity within a group.

In contrast, the *Gammaproteobacteria* represented only about 10% of total and BrdU-positive cells, but

the immunofluorescence intensities in this group (including the SAR86 and *Vibrio* groups) were higher than those of the *Rhodobacter/Roseobacter* and *Bacteroidetes* groups (ANOVA, Tukey-Kramer post hoc test, $p < 0.05$; Fig. 5). *Gammaproteobacteria* are not typically abundant in aquatic bacterial communities, but they have the potential for rapid growth (Eilers et al. 2000). In our study, *Gammaproteobacteria* were characterized as a less abundant but highly active subgroup of bacteria. Our results suggest that the activities of seawater bacterial assemblages are highly variable, depending on their phylotype composition and their population sizes.

The BrdU immunofluorescence intensity should be influenced by the genome size of individual cells. The genome size of marine bacteria varies at the species level, and ranges from 1.3 to 9 Mbp (Giovannoni et al. 2005). For example, the genome size of *Pelagibactor ubique* is 1.3 Mbp (Rappé et al. 2002), that of *Silicibacter pomeroyi* is 4.1 Mbp (Moran et al. 2004), and that of *Vibrio fischeri* is 4.3 Mbp (Ruby et al. 2005). These differences should be among the reasons why the immunofluorescence intensity varied among specific phylognetic groups (Fig. 5). Also, the multiple genomes during chromosome replication would cause the variation in immunofluorescence intensity among the cells. Thus, some sort of normalization including variety of the genome size and proliferation phase would be useful and necessary to calculate more precisely the growth rate from the immunofluorescence intensity.

The BrdU labeling efficiency should be a critical point in the BrdU methodology. In this study, the labeling efficiencies of the isolates excluding *Tenacibaculum* sp. and *Photobacterium* sp. were ~4% of their genomes (assuming a genome size of 3 Mb, double strands, one-quarter of DNA occupied by TdR or BrdU, 50% of GC content, 0.1 amol BrdU cell^{-1}). For nucleotide biosynthesis in cells, there are 2 principal pathways: (1) the de novo pathway, in which the nucleotides are synthesized from basic cellular components; and (2) the salvage pathway, in which free bases and nucleosides arising from breakdown of excess nucleotides or nucleic acid are converted back to nucleotide triphosphates (Moriarty 1986, Kornberg & Baker 1992). It is speculated that the contribution of the salvage pathway is lower than that of the de novo pathway for DNA synthesis in this experimental condition. Also, if thymidine is present in the incubation medium, bacterial cells preferentially take up the thymidine instead of BrdU (a BrdU:TdR incorporation ratio of 0.7, Steward & Azam 1999). These are probably the reasons why BrdU labeling efficiencies were low in this study. However, the method in its present level of sensitivity can be applied to assess the phylotype-specific growth characteristics in the environmental samples.

The growth characteristics of seawater bacterial assemblages have been assessed by methods that use radioisotope-labeled substrate incorporation. Microautoradiography-FISH has been recently used to assess phylotype-specific activities such as growth and substrate uptake at single-cell levels. The combination of BrdU immunocytochemistry and FISH is a non-radioisotopic alternative for assessing phylotype-specific growth characteristics. An advantage of this method is that it can be used in combination with other BrdU-based methodologies. Although FISH identifies only a broad range of phylotypes, detailed phylotypes of BrdU-incorporating bacteria can be determined by using BrdU immunocapture and 16S rRNA gene PCR-fingerprinting/cloning methods (Urbach et al. 1999, Yin et al. 2000, Hamasaki et al. 2007, Taniguchi & Hamasaki 2008). Also, bulk measurement of BrdU incorporation is an alternative to the conventional thymidine method for measuring bacterial productivity (Steward & Azam 1999, Nelson & Carlson 2005, Hamasaki 2006). Simultaneous use of these multiple BrdU-based methodologies provides a powerful tool for multi-scale comprehensive analyses of bacterial growth responses in seawater environments.

CONCLUSIONS

We successfully combined BrdU immunocytochemistry with FISH, optimizing the procedure for application to seawater bacterial assemblages. The method revealed the differences in BrdU incorporation rates and percentages in actively growing cells among various phylotypes. This method is applicable to spatio-temporal monitoring of bacterial growth in seawater and can reveal the relative contributions of major bacterial phylotypes to total bacterial productivity, as measured by TdR or BrdU incorporation. The method also has the potential to measure phylotype-specific growth rates at the single-cell level, which is promising for assessing microscale bacteria–bacteria or bacteria–particle interactions in natural environments.

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