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Fluorescence *in situ* hybridization of 16S rRNA gene clones (Clone-FISH) for probe validation and screening of clone libraries

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Summary

A method is presented for fluorescence *in situ* hybridization (FISH) of 16S rRNA gene clones targeting *in vivo* transcribed plasmid inserts (Clone-FISH). Several different cloning approaches and treatments to generate target-rRNA in the clones were compared. Highest signal intensities of Clone-FISH were obtained using plasmids with a T7 RNA polymerase promoter and host cells with an IPTG-inducible T7 RNA polymerase. Combined IPTG-induction and chloramphenicol treatment of those clones resulted in FISH signals up to 2.8-fold higher than signals of FISH with probe EUB338 to cells of *Escherichia coli*. Probe dissociation curves for three oligonucleotide probes were compared for reference cells containing native (FISH) or cloned (Clone-FISH) target sequences. Melting behaviour and calculated T_d values were virtually identical for clones and cells, providing a format to use 16S rRNA gene clones instead of pure cultures for probe validation and optimization of hybridization conditions. The optimized Clone-FISH protocol was also used to screen an environmental clone library for insert sequences of interest. In this application format, 13 out of 82 clones examined were identified to contain sulphate-reducing bacterial rRNA genes. In summary, Clone-FISH is a simple and fast technique, compatible with a wide variety of cloning vectors and hosts, that should have general utility for probe validation and screening of clone libraries.

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Introduction

Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeting 16S rRNA (DeLong *et al*., 1989; Amann *et al*., 1990b) has been widely used in environmental microbiology for two common applications: (i) to quantify certain phylogenetic groups with previously designed and tested probes (Amann *et al*., 1995), and (ii) to verify the occurrence of microorganisms of which 16S rRNA sequences were detected in clone libraries, and to investigate their distribution and abundance in the respective sample (Olsen *et al*., 1986). The latter application often includes the design of new, clone-specific oligonucleotide probes (Amann *et al*., 1995). Verifying the specificity of these new probes is often problematic due to the lack of pure cultures with identical probe target site. Hybridization conditions are usually tested either with *in vitro* transcribed, immobilized rRNA by membrane hybridization and subsequently adapted for FISH (Polz and Cavanaugh, 1997; Pernthaler *et al*., 1998), or roughly estimated by FISH directly in the environmental sample (Pernthaler *et al*., 2001).

A simple method is therefore desirable for directly validating probe specificity using FISH of cells containing the rRNA target sequence of interest. As transcripts of plasmids have been shown to be detectable by FISH under certain conditions (Juretschko *et al*., 1999), 16S rRNA gene clones containing the target sequence as insert should be suitable for FISH—if target-rRNA of appropriate quality and abundance can be generated in these clones. In addition, FISH of clones should also be useful for screening clone libraries for insert sequences of special interest.

Up to now, only anecdotal reports exist regarding the use of FISH targeting clones for probe testing (Simon *et al*., 2000) or the screening of a clone library (Tonolla *et al*., 2000); information about the technical details and a systematic evaluation of conditions to achieve maximum signal intensities have not been documented. The goal of this study was therefore to develop a standard FISH pro-

tocol for transcripts of specific plasmid inserts (referred to as Clone-FISH in this article), and to test its feasibility for probe validation and sorting of clone libraries.

Results and discussion

FISH of in vivo *transcribed plasmid inserts.*

Any application of FISH to clone-specific transcripts must fulfil two conditions to be useful for probe validation and screening of clone libraries: (i) signal intensities must be high enough for detection by epifluorescence microscopy and ideally flow cytometry; (ii) signals must originate from hybridization of *in vivo* transcribed rRNA, not of plasmid DNA, so that hybridization of clones is comparable to FISH of native cells. A common and strong promoter for *in vitro* transcription of plasmid inserts is the T7 RNA polymerase promoter present on all plasmids used in this study. For *in vivo* transcription from the T7 promoter, *Escherichia coli* host strains containing a genomic copy of the T7 RNA polymerase are required, e.g. the (DE3) strains utilized in this study. After induction of the T7 polymerase with isopropyl-β-D-thiogalactopyranoside (IPTG), transcription of the insert situated downstream of the T7 promoter should generate sufficient target for FISH.

Clones grown overnight as negative controls were not detectable by FISH with a transcript-specific probe except for very few cells per sample, which might be due to uneven distribution of multicopy plasmids (Novick, 1987) in combination with 'leaky transcription' from the T7 or lacZ promoter. In contrast, when clones were incubated with IPTG for 3 or 5 h, cells yielded detectable signals in all cloning approaches evaluated (except with plasmid pGEMEX-1), with some variations between different vectors and host cells (Tables 1 and 2). However, signal distribution within the cells was often patchy or concentrated at the cell ends, and only a fraction of the cells (typically <50%) showed detectable signals at all (Fig. 1A and B).

An alternative approach to generate high numbers of target rRNA is the use of chloramphenicol (Camp). Camp increases plasmid copy number (Sambrook *et al*., 1989) and leads to an accumulation of RNA (Vasquez, 1966), which should also encompass rRNA from leaky transcription of the plasmid insert (see above). Incubation of clones with Camp (170 mg l^{-1} or 1700 mg l^{-1}) for 1–5 h did not result in FISH signals above background levels, i.e. only a few cells per sample were detectable. However, when clones [non-(DE3) host strains, i.e. no T7 RNA polymerase present] were incubated with chloramphenicol overnight, weak but clearly visible signals were obtained (Fig. 1C and D). Though weaker than after IPTG-induction, signals were more evenly distributed in the cells, and typically >90% of cells were FISH-positive. *In vivo* tran-

Table 1. Signal intensities of FISH (estimated from microscopic observation) of all clone lines and treatments used.[a]

		Incubation								
			Chloramphenicol		IPTG[c]		IPTG + Chloramphenicol			
Plasmid	Host cells	None[b]	1–5 h[b]	Overnight	3 h	5 h	2 h	3 h	4 h	5 h
pGEM-T	JM109	–	–	ND				ND		
	JM109(DE3)	–	–	ND	(–)	(–)	++[c]	++[c]	++[c]	++[c]
	NovaBlue(DE3)	–	–	ND	(+)	(+)	++	++		+++
	TOP10F′	–	ND	+			ND		+++	
pCRII-TOPO	TOP10F′	–	ND	+			ND			
pGEM-3z	JM109	–	–	ND	ND					
	JM109(DE3)	–	–	ND	(+)	(+)	++	++	+++	+++
	NovaBlue(DE3)	–	–	ND	(++)	(++)	++[c]	++[c]	+++[c]	+++[c]
pGEMEX-1	JM109	–	–	ND			ND			
	JM109(DE3)	–	–	ND	–	–	–	–	–	–
	NovaBlue(DE3)	–	–	ND	–	–	–	–	–	–
pETBlue-1	NovaBlue	–	–	ND			ND			
	NovaBlue(DE3)	–	–	ND	+	++	+++	+++	+++	+++
	Tuner(DE3)	–	NA[e]	NA	(–)	(++)		NA		
	JM109(DE3)	–	–	ND	(+)	(++)	++	+++	+++	+++
pET-23(+)	NovaBlue	–	–	ND			ND			
	NovaBlue(DE3)	–	–	ND	(++)	(++)	+++	+++	+++	+++
	Tuner(DE3)	–	NA	NA	(+)	++		NA		
	JM109(DE3)	–	–	ND	(++)	(++)	++	+++	+++	+++

a. –, no signals; +, weak signals; ++, good signals; +++ very bright signals; sign in brackets, only a fraction (10–50%) of all cells hybridized.
b. In most samples, very few cells (<5%) had weak to good fluorescence.
c. Patchy distribution of fluorescence inside the cells, i.e. mostly concentrated at the cell ends.
d. ND, not determined.
e. NA, not applicable, because Tuner(DE3) is chloramphenicol-resistant.

Table 2. Relative fluorescence signal intensity [%][a] (quantified by flow cytometry) of selected clone treatments.

	Incubation conditions							
		Chloramphenicol	IPTG		IPTG + chloramphenicol			
Vector and host	None	5 h	3 h	5 h	2 h	3 h	4 h	5 h
pGEM-T in JM109	0	5	ND[b]	ND	ND	ND	ND	
pGEM-T in JM109(DE3)	ND	0[c]	4	2	48	31	74	67
pET-23(+) in NovaBlue(DE3)	0	0[c]	93[d]	74[d]	160	ND	182	183
pET-23(+) in JM109(DE3)	ND	0[c]	124[d]	81[d]	105	223	284	285

a. Signal intensities are displayed relative to FISH of *E. coli* cells with probe EUB338, and were corrected for background fluorescence (hybridization with non-binding control probe NON338).
b. ND, not determined.
c. Very few cells in each sample had low to medium signal intensities; signals were not quantified.
d. Signal intensity of only a fraction of all cells (10–50%); the remaining cells had signal intensities of 0–21% of the *E. coli*/EUB338 signal.

scribed rRNA (from leaky transcription of the plasmid insert even without IPTG-induction) was the primary target after Camp treatment, as was documented in three ways: (i) RNase treatment resulted in disappearance of signals (Fig. 1E and F); (ii) DNase treatment did not change the signal (data not shown); and (iii) FISH with a reverse complement probe (i.e. targeting the antisense strand of the plasmid but not the transcribed rRNA) gave no signals. Therefore, in the absence of IPTG-induction low intensity FISH signals derive from hybridization to *in vivo* transcribed rRNA, not to plasmid DNA.

Significantly brighter and more uniform signals were observed when Camp treatment was combined with IPTG-induction. Following combined treatment virtually all cells were probe-positive and individual cells showed uniform distribution of fluorescence (Fig. 1G). Signal intensity was comparable to or even higher than that of a standard FISH of *Escherichia coli* cells with probe EUB338 (Fig. 1G and H; Tables 2 and 3). Paraformaldehyde-fixed clones could be stored in PBS:ethanol (1:1) at –20°C for at least 12 months without significant loss of signal. As for the Camp-treated clones, RNase treatment led to the disappearance of signals, which again proves that *in vivo* transcribed rRNA has been hybridized (data not shown). Differences between the various plasmids and host cells were similar as reported above, and there were also differences related to the time of incubation (Table 1).

The three most promising cloning approaches were chosen for quantitative analysis of signal intensities after the various treatments, and the results are summarized in Table 2. In addition, identical treatments of five other vector-host combinations were included for comparison (Table 3). The brightest signals were obtained with vector pET-23(+) and host cells JM109(DE3), with a maximum intensity 2.8-fold higher than with log-phase *Escherichia coli* cells hybridized with probe EUB338. These data confirm the microscopic observation that a combination of IPTG-induction and Camp treatment is superior to IPTG-induction or Camp treatment alone. They also show that 3–4 h incubation with Camp following a 1-h IPTG-induction is sufficient for very high signals, and that signals do not increase after 4 h. Virtually all vectors (with the exception of pGEMEX-1) and host cells were shown to be suitable for FISH of *in vivo* transcribed plasmid inserts. However, if high signal intensities are desired, the use of vectors with T7 promoters and host cells with IPTG-inducible T7 RNA polymerase is recommended, and chloramphenicol-resistant host strains need to be avoided.

Standard protocol for Clone-FISH

From the comparison of different treatments, the following standard protocol for preparation of clones for FISH is suggested: (i) select appropriate vector–host combination [e.g. pGEM-T in JM109(DE3) (for easy A-T-cloning of PCR products) or pET-23(+) in NovaBlue(DE3) (for blunt- or sticky-end cloning)]; (ii) grow to OD_{600} of 0.3–0.4 from 1:1000 dilution of overnight culture; (iii) add IPTG (1 mM) and incubate for 1 h; (iv) add chloramphenicol (170 mg l^{-1}) and incubate for 4 h; (v) fix with paraformaldehyde and store in PBS:ethanol (1:1) at –20°C (Pernthaler *et al.*, 2001). Clones can be used for FISH for

Table 3. Relative fluorescence signal intensity [%][a] (quantified by flow cytometry) of different clone lines after incubation with IPTG + chloramphenicol (3 h).

Vector and host	Relative signal intensity (%)
pGEM-T/JM109(DE3)	31
pGEM-T/NovaBlue(DE3)	80
pGEM-3z/JM109(DE3)	127
pGEM-3z/NovaBlue(DE3)	32
pETBlue-1/NovaBlue(DE3)	87
pETBlue-1/JM109(DE3)	75
pET-23(+)/JM109(DE3)	223

a. Relative to FISH of *E. coli* with probe EUB338 (see Table 2).

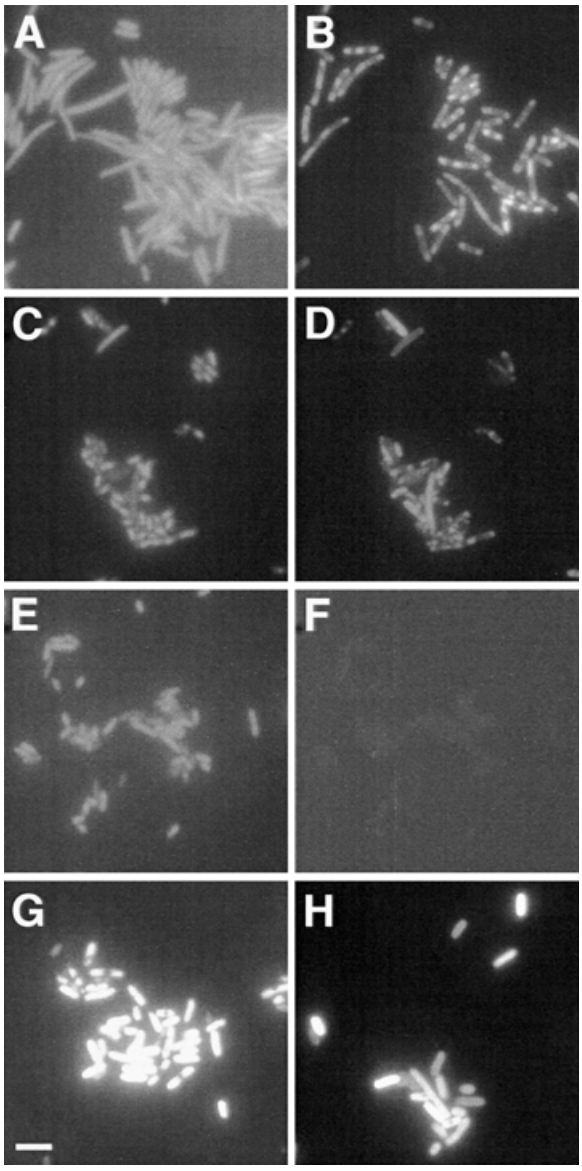

Fig. 1. Epifluorescence micrographs of clones after various treatments (vector/host combination in brackets): IPTG-induction, 3 h [pET-23(+)/NovaBlue(DE3)] (A and B); chloramphenicol treatment overnight (pGEM-T/TOP10F′) (C and D); chloramphenicol treatment overnight plus RNase treatment (pGEM-T/TOP10F′) (E and F); and IPTG-induction combined with chloramphenicol treatment, 4 h [pET-23(+)/JM109(DE3)] (G). DAPI-staining (A, C and E) and FISH (B, D and F) of identical microscopic fields are shown. FISH of *Escherichia coli* cells with probe EUB338 is shown for comparison of signal intensity (H). All FISH images were taken at a fixed exposure time of 1.8 s. Scale bar, 5 μm (applies to all images).

more than one year; the only probes that are suitable for Clone-FISH do not target rRNA of the *E. coli* host cells.

Use for probe validation

Our documentation that fluorescence is conferred by hybridization of probe to the *in vivo*-transcribed insert RNA offers the possibility to use clones instead of native cells to validate probe specificity and optimize hybridization conditions, provided that hybridization to the transcript and rRNA of native cells is comparable. To test comparability, melting curves [i.e. signal intensities of FISH at increasing formamide concentrations to simulate increasing hybridization temperatures (Pernthaler *et al*., 2001)] of 16S rRNA gene clones and corresponding pure cultures of three phylogenetic groups (*Pseudomonas aeruginosa* and *Nitrosomonas europaea*, γ- and β-subgroups of Proteobacteria respectively; *Paenibacillus polymyxa*, Firmicutes) were compared (Fig. 2). Although fluorescence intensities were slightly different for clones and cells, their melting behaviours were similar and the calculated T_d values virtually identical to native transcripts (Table 4). It should be noted that we have so far examined only a limited selection of phylogenetic groups and further studies are required to establish comparability for more disparate groups. (e.g. Archaea). However, our data indicate that this approach is generally applicable and that clones can be utilized, instead of reference strains, to test newly designed probes. This might be especially useful when pure cultures are very slow or difficult to grow, or in the absence of any reference cultures (e.g. when designing specific probes for clones or groups of uncultivated organisms that are only represented by clone sequences). Clone-FISH therefore offers a convenient alternative to membrane hybridization of *in vitro*-transcribed rRNA (Polz and Cavanaugh, 1997; Pernthaler *et al*., 1998).

Screening of clone libraries.

A 16S rRNA gene clone library was constructed from bacterioplankton samples of the chemocline of meromictic Lake Cadagno in standard, non-(DE3) host cells TOP10F′ with vector pGEM-T. Following Camp-treatment overnight, 13 clones were FISH-positive (i.e. gave higher signal intensities than with the non-binding control probe NON338) from 82 clones examined using probe SRB385 specific for sulphate-reducing bacteria (SRB). Full- or partial-length sequencing of the clone inserts confirmed the phylogenetic affiliation of the FISH-positive clones with SRB (EMBL accession no. AJ389622-29; Tonolla *et al*., 2000), whereas the remaining 69 clones belonged to

Table 4. T_d values of probes PS224, Nso1225, and LGC354B for FISH with either native cells or clones with heterologous expression of the respective 16S rRNA genes (calculated from Fig. 2).

Probe	FISH of cells	FISH of clones
PS224	67.5°C	67.5°C
Nso1225	63.5°C	63.5°C
LGC354B	65.5°C	66.5°C

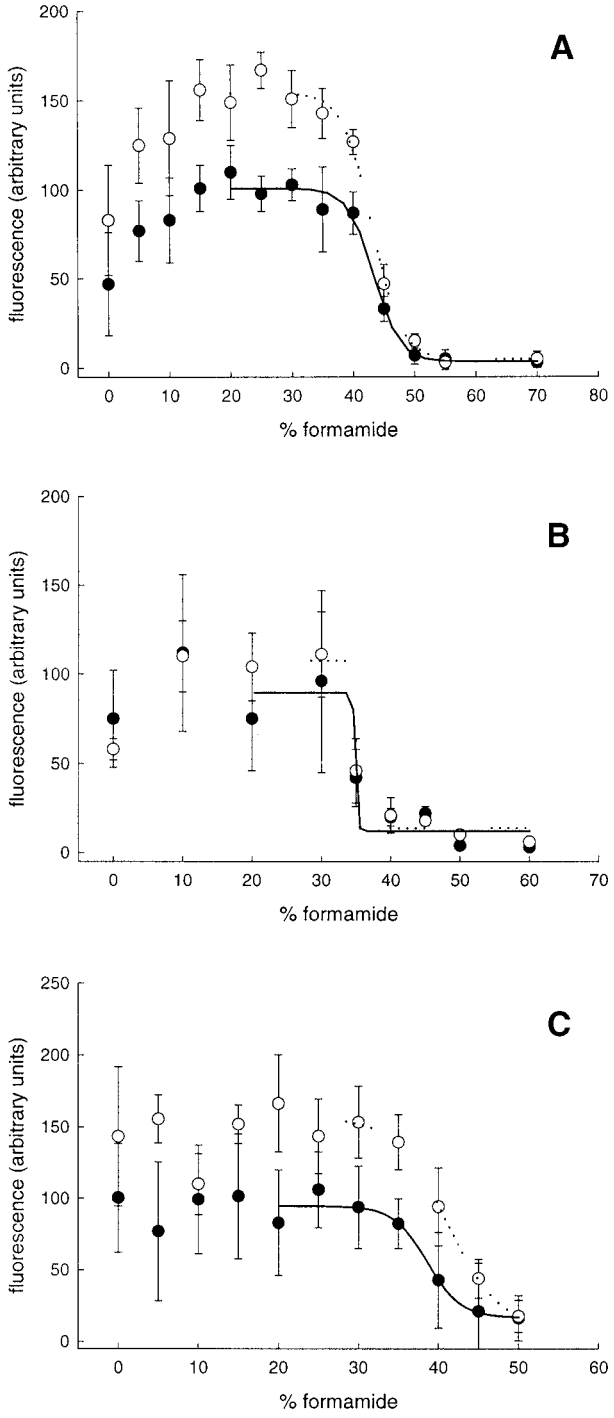

Fig. 2. Probe dissociation curves of probes PS224 (A), Nso1225 (B) and LGC354B (C), with cells of *P. aeruginosa* (A), *N. europaea* (B) *and P. polymyxa* (C) (closed circles), in comparison to probe dissociation curves with the respective 16S rRNA gene clones (open circles). Solid and dotted lines represent sigmoidal fittings of data points from cells and clones respectively.

other phylogenetic groups (data not shown). Thus, the lower signal intensities obtained without IPTG-induction were sufficient for a reliable screening of this clone library. Consequently, by using Camp-treatment of non-(DE3) host cells, it is possible to combine standard blue-white screening with Clone-FISH. This method appears to be an easy and cost-efficient alternative to conventional screening procedures, which usually include plasmid extractions, PCR, restriction digestion or membrane hybridizations. In addition, it might be combined with flow cytometric sorting of FISH-positive clones for fast- and high-throughput screening.

Conclusion

FISH targeting rRNA that has been transcribed *in vivo* from a vector insert (Clone-FISH) is an easy and fast technique, possible with a wide variety of cloning vectors and hosts. We anticipate that it will be useful for probe validation and screening of clone libraries. In combination with the sorting capacities of modern flow cytometers, rapid screening and sorting of large clone libraries even for clones of very low abundance might become possible.

Experimental procedures

Bacterial strains

Pseudomonas aeruginosa PAO1, *Nitrosomonas europaea* ATCC19718 and *Paenibacillus polymyxa* ATCC8523 were harvested in mid-log phase. One aliquot was fixed for whole-cell hybridizations with paraformaldehyde (*P. aeruginosa, N. europaea*) or ethanol (*P. polyyxa*) according to published protocol (Pernthaler *et al*., 2001), whereas another aliquot was used for extraction of DNA with a commercial kit (QIAgen DNA Minikit, Qiagen Inc., Valencia, CA). The following *Escherichia coli* strains were used as host cells for the various cloning approaches: JM109(DE3), JM109 (both Promega, Madison, WI); Tuner(DE3), NovaBlue(DE3), NovaBlue (all Novagen, Madison, WI); and TOP10F′ (Invitrogen, Carlsbad, CA). *Escherichia coli* DMS 30083T was used as a positive control and for the normalization of signal intensities in the FISH experiments.

PCR amplification and cloning

Near full-length 16S rRNA genes were amplified in standard PCR reactions (50 μl) containing approximately 100 ng of template DNA, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet®P40, 1.5 mM Mg^{2+}, 200 μM of each dNTP, 50 pmol of each primer, and 2.5 U Taq polymerase. Annealing temperature was 45°C, numbers of cycles 30, and primer pairs were GM3/G4 (Muyzer *et al*., 1995) for *P. aeruginosa* and *N. europaea*, and 27F/1492R (Lane, 1991) for *P. polymyxa*. PCR products of *P. aeruginosa* were used for testing different cloning and incubation strategies; the resulting standard protocol (see *Results and discussion* section) was then

applied to PCR products of *N. europaea* and *P. polymyxa*. PCR products of *P. aeruginosa* were either directly ligated into cloning vectors pGEM-T (Promega), or blunt-ended using a commercial endconversion mix (Novagen) and ligated into blunt-ended, dephosphorylated vectors pGEM-3z, pGEMEX-1 (both Promega), pET-23(+) or pET-Blue-1 (both Novagen) that had been prepared by restriction digestion and alkaline phosphatase treatment following standard protocols (Sambrook *et al*., 1989). Ligation reactions and subsequent transformations into chemically competent cells were as recommended by the respective manufacturers. Where applicable [only for non-(DE3) host cells containing pGEM-T, pGEM-3z, or pET-Blue-1], standard blue-white screening of clones preceded PCR-screening for insert content and insert direction. For the latter, standard PCR reactions with an annealing temperature of 50°C and 30 cycles were performed with vector-specific forward primers [pUC/M13f (Promega) or T7 promoter primer (Novagen)] and the oligonucleotide probes that were intended for FISH (see below) as insert-specific reverse primers. Clones with the insert in a forward direction, i.e. which would yield rRNA upon transcription with T7 DNA polymerase, were selected and routinely maintained on LB plates containing the appropriate antibiotics for the respective vectors and host cells.

Environmental clone library

Water samples were obtained from the chemocline of meromictic Lake Cadagno, and bacterioplankton was collected as decribed previously (Demarta *et al*., 1998). Extraction of genomic DNA and amplification of almost full-length bacterial 16S rRNA gene fragments was according to published protocol (Tonolla *et al*., 1999). Fragments were subsequently ligated into plasmids pGEM-T (Promega) or pCRII-TOPO (Invitrogen), and were transformed into TOP10F′ (Invitrogen) following the manufacturer's instructions. Clones were incubated and hybridized as described below. Clones showing signals after FISH with probe SRB385 were selected for various incubation treatments (chloramphenicol, RNase, DNase; see below) and for further analysis of the insert by reamplification, sequencing, and phylogenetic analysis as described previously (Tonolla *et al*., 1999).

Clone incubation in preparation for FISH

All incubations were done at 37°C on a shaker. Overnight cultures of clones in LB broth (plus antibiotics) were diluted 1:1000, and grown to an OD_{600} of 0.3–0.4. Then, the *in vivo* transcription of the rRNA gene inserts was induced by addition of 1 mM IPTG, and samples were fixed for FISH with paraformaldehyde after 3 and 5 h of incubation. Alternatively, diluted overnight cultures were grown to an OD_{600} of 0.6, chloramphenicol (170 mg l^{-1} or 1.700 mg l^{-1}) was added (Sambrook *et al*., 1989), and samples were fixed every hour for 5 h. For a long-term chloramphenicol treatment, cells that had reached OD_{600} of 0.6 were diluted again (1:20); after exactly 2.5 h ($OD_{600} \sim 0.4$) chloramphenicol was added (170 mg l^{-1}), and the culture was incubated overnight.

Finally, the short-term chloramphenicol treatment was combined with IPTG-induction, i.e. chloramphenicol (170 mg l^{-1}) was added 1 h after the IPTG-induction. Samples were fixed 2, 3, 4 and 5 h after the addition of chloramphenicol.

RNase and DNase treatment of incubated clones

RNA or DNA was removed from paraformaldehyde-fixed clones immobilized on gelatin-coated microscopic slides after dehydration in an ethanol series by adding DNase-free RNase (0.5 mg ml^{-1}) or RNase-free DNase (10 U ml^{-1}), respectively (both Boehringer Mannheim, Germany). Slides were incubated in a humid chamber at room temperature for 30 min and 2.5 h for RNase- and DNase-treatment respectively. Subsequently, four consecutive washes in phosphate-buffered saline [PBS; 130 mM NaCl, 10 mM sodium phosphate (pH 8.4)] were performed to stop the incubation and remove the nucleases (Tolker-Nielsen *et al*., 1997).

FISH of immobilized cells

For qualitative analysis of the different cloning strategies and incubations, standard FISH of immobilized cells was performed (Pernthaler *et al*., 2001), i.e. immobilization of fixed clones on slides, dehydration and hybridization with Cy3-labelled oligonucleotide probes in the standard hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.2, 0.01% SDS) containing 20% formamide. Probes were PS224 (5′-CCG ACC TAG GCT CAT CTG-3′) for *P. aeruginosa* 16S rRNA gene clones, SRB385 (Amann *et al*., 1990a), and a reverse complement probe of SRB385 (5′-CCT GAC GCA GCG ACG CCG-3′) for the environmental clone library. Probes EUB338 (Amann *et al*., 1990a) and NON338 (Manz *et al*., 1992) were used as positive and negative controls respectively. Hybridization time was 90 min at 46°C. Washing was in washing buffer [225 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.01% SDS] for 20 min at 48°C, and cells were counterstained with 4′6-diamino-2-phenylindole (DAPI; 0.5 μg ml^{-1}). Slides were evaluated using an Axioskop epifluorescence microscope (Carl Zeiss, Jena, Germany) with filter sets 01 and HQ-CY3, and images were captured with a charge-coupled device camera (CF 8/1 FMC, Kappa, Gleichen, Germany) using the Q500MC Image Processing and Analysis System (Leica Cambridge Ltd, Cambridge, UK).

FISH and flow cytometry

Signal intensities of selected samples were quantified by flow cytometry as described previously (Fuchs *et al*., 1998). In brief, fixed, suspended clones (approximate conc. 10^6 cells μl^{-1}) were hybridized at 46°C for 2 h in 80 μl of standard hybridization buffer containing 20% formamide and 2.5 ng μl^{-1} of fluorescein-labelled probe PS224. Subsequently, cells were pelleted by centrifugation for 2 min at 4000 × g and resuspended in 100 μl of hybridization buffer containing no probe. After washing for 30 min at 46°C, samples were mixed with 200 μl of PBS (pH 9.0), immediately placed on ice and analyzed within 1 h on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, California, USA). The parameters SSC (right-angle light scatter) and FL1 (fluores-

cein fluorescence) were recorded as pulse height signals (four decades in logarithmic scale each), and for each measurement 10 000 events were stored in list mode files. Subsequent analysis was done with the CellQuest software (Becton Dickinson, Mountain View, California). Probe-conferred fluorescence was determined as the median of the FL1-values of single cells lying in a gate that was defined in a SSC vs. FL1 dotplot. Fluorescence of cells was standardized to green fluorescent, 0.5 μm polystyrene beads (Polysciences, Warrington, USA). All measurements were done in triplicate, with each replicate representing an independent cell preparation and hybridization. Coefficient of variation of the triplicates was in all cases <10%.

Melting curve comparison

FISH of fixed cells of *P. aeruginosa, N. europaea* and *P. polymyxa* was compared with FISH of their respective 16S rRNA gene clones [vector: pGEM-T; host: JM109(DE3)] that had been treated by the standard protocol (see *Results and discussion* section). The following Cy3-labelled oligonucleotide probes (Qiagen Operon, Alameda, CA) were used: PS224; LGC354B (Meier *et al*., 1999); and Nso1225 (Mobarry *et al*., 1996). Dissociation studies were done with immobilized cells at a fixed hybridization temperature of 46°C by adding increasing concentrations of formamide to the standard hybridization buffer, assuming an increase of the effective hybridization temperature of 0.5°C per 1% of added formamide (Stahl and Amann, 1991). The fluorescence intensities after FISH were determined by image analysis from a series of images recorded by confocal laser scanning microscopy on a LSM510 (Carl Zeiss, Jena, Germany) at identical scanning parameters. The signal intensity of at least 200 single cells in 20 microscopic fields was determined using the IP Laboratory SPECTRUM software (version 2.5.5; Signal Analytics Corporation, Vienna, VA). Temperatures of dissociation (T_d) were determined as formamide concentrations from sigmoidal fittings of the intensity data with the software package ORIGIN (Microcal, Northampton, MA), and were then transformed to temperatures (see the relation above).

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