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The Domain-specific Probe EUB338 is Insufficient for the Detection of all *Bacteria*: Development and Evaluation of a more Comprehensive Probe Set

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Summary

In situ hybridization with rRNA-targeted oligonucleotide probes has become a widely applied tool for direct analysis of microbial population structures of complex natural and engineered systems. In such studies probe EUB338 (AMANN et al., 1990) is routinely used to quantify members of the domain *Bacteria* with a sufficiently high cellular ribosome content. Recent reevaluations of probe EUB338 coverage based on all publicly available 16S rRNA sequences, however, indicated that important bacterial phyla, most notably the *Planctomycetales* and *Verrucomicrobia*, are missed by this probe. We therefore designed and evaluated two supplementary versions (EUB338-II and EUB338-III) of probe EUB338 for *in situ* detection of most of those phyla not detected with probe EUB338. *In situ* dissociation curves with target and non-target organisms were recorded under increasing stringency to optimize hybridization conditions. For that purpose a digital image software routine was developed. *In situ* hybridization of a complex biofilm community with the three EUB338 probes demonstrated the presence of significant numbers of probe EUB338-II and EUB338-III target organisms. The application of EUB338, EUB338-II and EUB338-III should allow a more accurate quantification of members of the domain *Bacteria* in future molecular ecological studies.

Key words: Bacteria-specific probes – EUB338 – fluorescent *in situ* hybridization – digital image analysis

Introduction

A central aspect in microbial ecology is the identification and quantification of those microorganisms that occur in the examined habitats. This task is far from trivial because lack of distinctive morphological traits of most prokaryotes hampers their direct microscopic identification (WOESE, 1987). Cultivation approaches do, due to limited knowledge of nutritional and other growth requirements for the majority of microorganisms, only offer a limited and biased inventory of the naturally existing microbial diversity (for example WAGNER et al., 1993). The advent of rRNA-targeted oligonucleotide probe-based quantitative dot blot and fluorescent *in situ* hybridization (FISH) techniques allows for cultivation-independent insights into community structure and dynamics in microbial ecology (WAGNER et al., 1994; AMANN et al., 1995). Species-, genus-, group- and domain-specific probes were designed and successfully applied to study microbial consortia present in various natural and engineered systems (e.g. MANZ et al., 1992; RASKIN et al., 1994; WAGNER et al., 1994; GLÖCKNER et al., 1996; HARMSEN et al., 1996; MOBARRY et al., 1996; ZARDA et al., 1997; FELSKE et al., 1998; JURETSCHKO et al., 1998; LLOBET-BROSSA et al., 1998; MØLLER et al., 1998; MOTER et al., 1998; NEEF et al., 1998; MEIER et al., 1999). In such studies probe EUB338 (AMANN et al., 1990), which binds to the highly conserved region 338-355 (*E. coli* numbering) of the 16S rRNA molecule, has been generally used to quantify the domain *Bacteria*.

Recent studies indicated that at least one group of bacteria that is increasingly realized to be environmentally important, the *Planctomycetales*, are not detected by EUB338 (ZARDA et al., 1997; NEEF et al., 1998). Probe EUB338 was designed in 1990 (AMANN et al., 1990). Since then extensive comparative 16S rRNA sequence analysis has enormously improved our perspective on prokaryotic diversity. Several new phyla and divisons

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within the bacterial domain were recognized as being widely distributed in the environment (for a review see HUGENHOLTZ et al., 1998a). Future studies in microbial ecology should not ignore, but rather track those poorly, if at all characterized bacteria. Therefore, we reevaluated the coverage of all currently available 16S rRNA sequences of bacterial origin by probe EUB338 and developed (and evaluated) two modified versions of probe EUB338 which allow, if used in addition to EUB338, for a more complete detection of *Bacteria*.

Materials and Methods

Reference organisms, culture conditions, cell fixation, and biofilm sampling: All reference strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *Bacillus stearothermophilus* (DSM 22) was grown overnight in modified HD Medium (1% (w/v) tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose, 0.7% (w/v) NaCl, pH 7.4) at 55°C under agitation. *Verrucomicrobium spinosum* (DSM 4136) and *Planctomyces limnophilus* (DSM 3776) were cultured as described previously (HIRSCH and MÜLLER, 1985; SCHLESNER, 1987). Cultures were prepared for *in situ* hybridization by washing them once in phosphate buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer pH 7.2) and fixing them for three hours in paraformaldehyde (Sigma, Deisenhofen, Germany) as described by AMANN (1995) with the exception that the final paraformaldehyde concentration was 2% (w/v). *Bacillus stearothermophilus* was not fixed in paraformaldehyde but instead the cells were transferred to a 1:1 mixture of PBS and 96% ethanol (v/v) immediately after the washing step (ROLLER et al., 1994). Following the fixation, paraformaldehyde-fixed cells of the other species were washed once again in PBS and were then resuspended in the PBS:ethanol mixture, too. Paraformaldehyde-fixed cells of the *Parachlamydia*-like endosymbiont of *Acanthamoeba* spec. UWE25 (FRITSCHE et al., submitted) were kindly provided by MATTHIAS HORN, Technische Universität München. All fixed cultures were stored at –20°C. Expanded clay beads covered by nitrifying biofilm were sampled from a pilot-scale reactor treating municipal sewage (500 population equivalents, Biofor2, Ingolstadt, Germany). For fixation they were incubated for five hours in 3% (w/v) paraformaldehyde (final concentration) immediately after sampling. Subsequently, the biofilm was detached by manually swirling the beads within the fixative. Loosened biofilm was collected by centrifugation, resuspended in a 1:1 mixture of PBS and 96% (v/v) ethanol and stored at –20°C.

Oligonucleotide probes: The software package ARB (STRUNK and LUDWIG, http://www.biol.chemie.tu-muenchen.de/pub/ARB/) was used to evaluate the coverage of probe EUB338 (AMANN et al., 1990) on all 16S rRNA sequences in the TUM 16S rRNA ARB database (December 1998 database release, including approx. 10.500 bacterial and 500 archaeal 16S rRNA sequences) using the probe match tool. The specificity of the newly designed probes EUB338-II and EUB338-III was checked with the same program and database. For *in situ* hybridization, the probes were labeled at the 5'-end with the dye FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimide ester) or with one of the sulfoindocyanine dyes Cy3 and Cy5. When used as competitors in the probe dissociation experiments, the probes were applied as unlabeled oligonucleotides. Labeled and unlabeled probes were obtained from MWG (Ebersberg, Germany) or Interactiva (Ulm, Germany). Probe sequences and conditions required for specific hybridization are specified in the Results section.

***In situ* hybridization, DAPI-staining, and microscopy:** Fixed reference cells were spotted onto microscopic slides (Paul Marienfeld GmbH, Bad Mergentheim, Germany). For immobilization of biofilm samples gelatin-coated slides were used (AMANN, 1995). Slides were dried at 46 °C for 10 min and dehydrated in 50, 80 and 96% (v/v) ethanol. Whole cell and *in situ* hybridization experiments were performed as described previously (MANZ et al., 1992). Final probe concentrations in the hybridization buffer were 3 ng · μl^{-1} for probes labeled with Cy3 or Cy5, and 5 ng·μl^{-1} for probes labeled with FLUOS. Competitor oligonucleotide probes were used in equimolar amounts. Optimization of hybridization conditions for the newly developed probes EUB338-II and EUB338-III was performed as described by MANZ et al. (1992) using different concentrations of formamide and sodium chloride in the hybridization and wash buffers, respectively. Probe dissociation curves were obtained by digital image analysis of signal intensities of reference strains after *in situ* hybridization at different stringencies (see below). Following *in situ* hybridization, biofilm samples were stained at room temperature for five minutes with a 0.1% (w/v) solution of the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) (Sigma, Deisenhofen, Germany) in order to visualize all cells present in the sample regardless of their EUB338 target site sequences. Excess DAPI was removed with distilled water and the slides were air-dried. Prior to microscopic observation, slides were embedded in Citifluor (Citifluor, Canterbury, U.K.). Images were recorded using a LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with one UV laser (351 and 364 nm), one Argon laser (450 to 514 nm) and two Helium-Neon lasers (543 and 633 nm, respectively). For image reconstruction and printing the software package delivered with the microscope (Zeiss LSM 5 version 2.01) was used together with a Kodak 8650 PS printer.

Quantification of single cell fluorescence: Following *in situ* hybridization of suitable reference cells, for each probe and hybridization condition series of images of fluorescent cells were recorded by confocal laser scanning microscopy applying identical scanning parameters. Parameter settings were such that the brightest cell signals observed did not cause saturation (pixel grey values < 255) while low signal intensities (after applying highly stringent hybridization conditions) were still detectable. Subsequently, the intensity of single cell fluorescence was determined using the Zeiss Kontron KS400 image processing software (version 3.0) together with a newly developed macro (S.I.M., Signal Intensity Measurement). In brief, the first step performed by this macro is the mapping of all color information in an image to grey values of corresponding intensities. The resulting grey scaled image is the so-called "density image". Thereupon another grey scaled version of the original image is calculated, but this time with enhanced pixel intensities. This so-called "mask image" allows the user to spot even the darkest objects on the screen. The user then manually selects the smallest and largest cells in the "mask image" in order to define threshold values for the size range (in this case, the pixel area) of single cells. After the program has automatically selected all objects in the image with pixel areas between these thresholds, remaining artifacts can be manually marked for exclusion from further processing. At this point, special care must be taken to eliminate clustered cells, because they appear brighter than corresponding single cells. Thereafter the program measures the mean brightness of the selected cells in the "density image". This yields one mean intensity value for each cell. These values were imported automatically into suitable spreadsheet software (e.g. Excel, Microsoft, Redmond, Wa) for further processing and graphical visualization. The developed macro is available upon request.

Results

Coverage of currently available bacterial 16S rRNA sequences by probe EUB338

The TUM ARB sequence database, currently containing more than 10.500 bacterial 16S rRNA sequences, was screened to identify those bacteria displaying mismatches within the 18 nucleotide stretch (*E. coli* positions 338-355) representing the probe EUB338 target site. Despite the presence of probe EUB338 target site in 97% of the bacterial 16S rRNA sequences, this search revealed that only 6 of its 18 nucleotides (*E. coli* positions 338, 343, 346, 352, 354, 355) are completely conserved throughout the domain *Bacteria*. None of these represents a signature for the domain *Bacteria*, since identical nucleotides are found at the respective positions in the domains *Archaea* and/or *Eucarya*.

In addition to probe EUB338, other rRNA targeted oligonucleotide probes for detection of the domain *Bacteria* have been published (for a list of domain specific probes, refer to AMANN et al., 1995). Screening of the TUM ARB database for those sequences which are fully complementary to each of these probes revealed most encompassing coverage of the domain *Bacteria* by probe EUB338. Therefore we decided to restrict our further analyses to probe EUB338 which offered the best starting-point for the design of a more comprehensive probe set.

Different types of sequence variations within the probe EUB338 target site could be detected for sequences

Table 1. 16S rRNA sequences with variations at the target site of probe EUB338 that are different from sequence variation types I–III. For environmentally-derived cloned sequences accession numbers are given.

Phylogenetic division/organisms or clones	EUB338 target site sequence variation original EUB338 target site: ACUCCUACGGGAGGCAGC
Aquificales	
1) *Aquifex pyrophilus, Calderobacterium hydrogenophilum, Hydrogenobacter thermophilus, H. acidophilus, Thermocrinis ruber,* clones NAK14 (AB005738), OPB13 (AF027098), OPS1 (AF018186), OPS4 (AF018189), OPS5 (AF018190), OPS6 (AF018191), OPS7 (AF018192), OPS10 (AF018194), OPS14 (AF018196), strEM_17 (U05661)	--C--------G------
2) clone GANI4 (AB005736)	--AA-------G------
Candidate division OP11	
clone LGd14 (AF047573)	--AU------AU------
Green non-sulfur bacteria	
1) *Herpetosiphon geysericola, H. aurantiacus*	----------G------
2) clones SJA-35 (AJ009460), WCHB1-50 (AF050571)	--G--------U------
High GC Gram positive	
Prauseria hordei, Nocardiopsis alba, Thermomonospora alba, clones 56AT148 (Z73374), 56AT1203 (Z73373)	------G----------
Acidobacterium/Holophaga	
clones "unidentified bacterium 29" (AB004577), "unidentified bacterium 30" (AB004578)	-------U----------
Chlamydiales	
Chlamydia psittaci	---U------A----U--
Low GC Gram positives	
1) *Mycoplasma iowae, M. muris, M. penetrans, M. volis, Eubacterium timidum, Spiroplasma* group IX (M24474)	---U------A-------
2) *Eperythrozoon suis, Haemobartonella felis, H. muris*	-U----------A-----
3) *Haemaphysalis longcornis*	----U----A--------
4) clones RF8 (AF001741), RF9 (AF001742), RF39 (AF001770)	----------A----
Spirochetes	
1) *Critispira pectinis*	--CA------UG------
2) *Treponema maltophilum*	---U--------------
Proteobacteria	
1) *Orientia tsutsugamushi*	----------G------
2) clones Alpha9 (L35460), Alpha12 (L35465)	---U------A-------

affiliated with the phyla *Aquificales*, Spirochetes, green non-sulfur bacteria, Gram-positive bacteria with low and high G+C content of the DNA, *Planctomycetales*, *Chlamydiales*, *Verrucomicrobia*, *Acidobacterium/Holophaga*, *Cytophaga/Flavobacterium/Bacteroides* (CFB), *Proteobacteria*, and the candidate phylum OP11 (Figure 1, Table 1). Among these phyla probe EUB388 coverage is still almost complete for the *Proteobacteria* (mismatches are found only for 3 sequences affiliated to the alpha-subclass), the *Cytophaga/Flavobacterium/Bacteroides* phylum (a single partial sequence of a molecular isolate from activated sludge does not possess the target site), the *Acidobacterium/Holophaga* phylum (only two molecular isolates differ within the probe EUB338 target site), and the Spirochetes (*Treponema maltophilum* and *Critispira pectinis* have mismatches to EUB338). We cannot exclude that these gaps in otherwise well covered groups originate from sequencing errors. Higher numbers of representatives lacking full complementarity to EUB338 were found among the Gram-positive bacteria with low and high G+C content of the DNA and green non-sulfur bacteria phyla as well as the candidate phylum OP11. Furthermore, none of the sequences affiliated to the phyla *Planctomycetales*, *Aquificales*, *Chlamydiales,* and *Verrucomicrobia* possess a target region fully complementary to probe EUB338.

In the following paragraph we present a detailed description of sequence variations of the probe EUB338 target site for the phyla *Planctomycetales*, *Chlamydiales,* and *Verrucomicrobia*. Within the *Planctomycetales*, two types of sequence variation at the *E. coli* positions 338-355 were observed. Sequence variation type I, represented by the genus *Isosphaera*, is characterized by an A → U transversion at *E. coli* position 353. This sequence variation is not restricted to the *Planctomycetales* but is also found for all but one member of the *Chlamydiales*, some representatives of the Gram-positive bacteria with low G+C content of the DNA, three molecular isolates of the *Verrucomicrobia* phylum, and one molecular isolate of candidate phylum OP11 (Fig. 1A). Sequence variation type II, characterized by an A → U transversion at *E. coli* position 353, an A → U transversion at *E. coli* position 349, and a U → A transversion at *E. coli* position 340, is with exception of the genus *Isosphaera*, present in all other *Planctomycetales* and in two environmentally derived 16S rDNA clones affiliated to candidate phylum OP11, but is absent from other bacteria in the database. Concerning the *Chlamydiales*, as mentioned above, all members except for *Chlamydia psittaci*, do possess sequence variation type I. Within the phylum *Verrucomicrobia* a third type of sequence variation of *E. coli* positions 338-355 was identified. Sequence variation type III differs from the probe EUB338 target site due to a U → A transversion at *E. coli* position 340, and an A → U transversion at *E. coli* position 349. This sequence variation type is found in all but three 16S rRNA sequences belonging to the *Verrucomicrobia* and is also present in some members of the green non-sulfur bacteria, and in the only partially sequenced molecular isolates SMKN41 and clone SBR1082 tentatively clustering together with the Gram-positive bacteria with high G+C content of the DNA and the CFB, respectively. Figure 1A summarizes the distribution of the EUB338 target sequence and of the three above described types of variations among the phyla of the domain *Bacteria*.

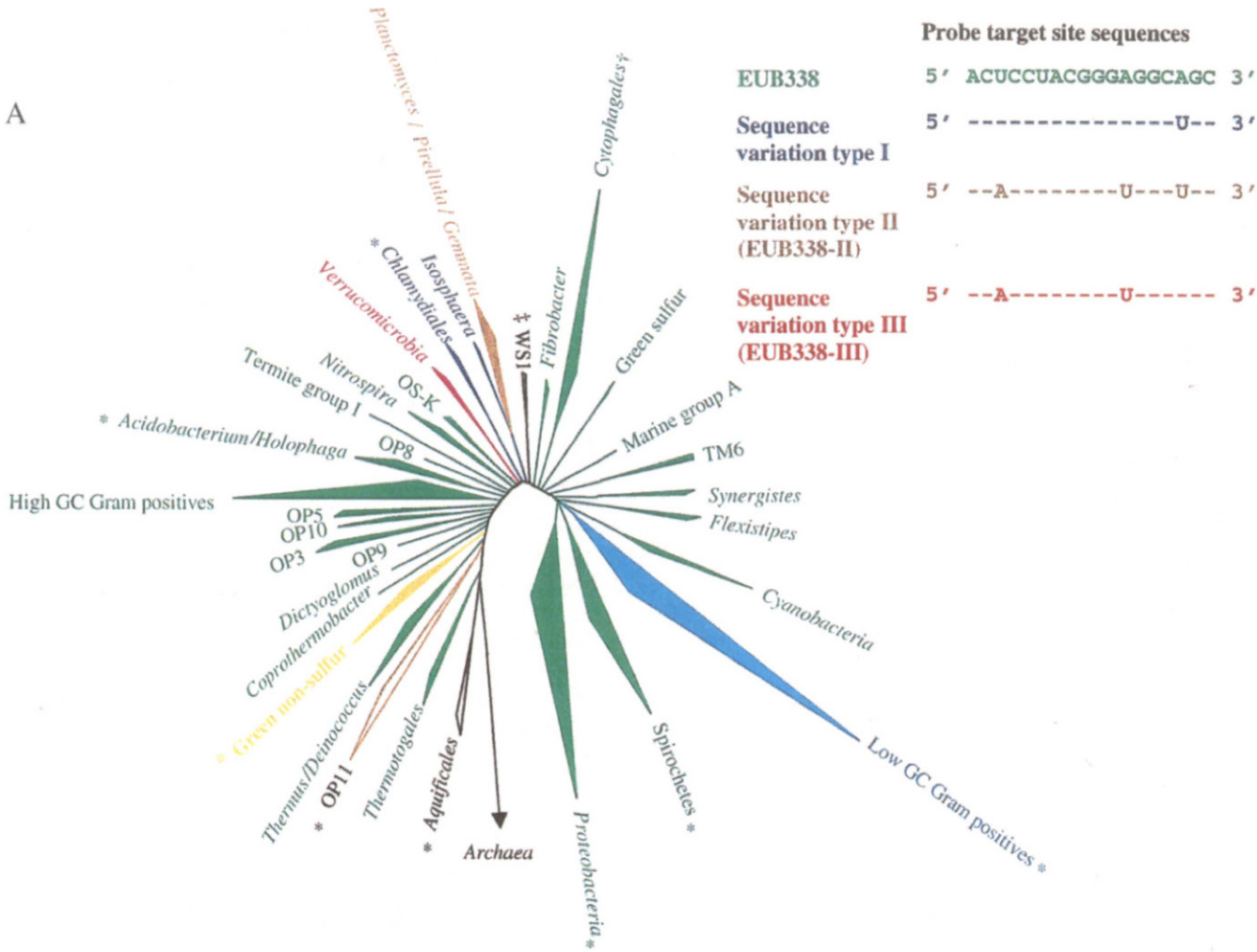
Experimental evaluation of the detection of *Bacteria* by probe EUB338

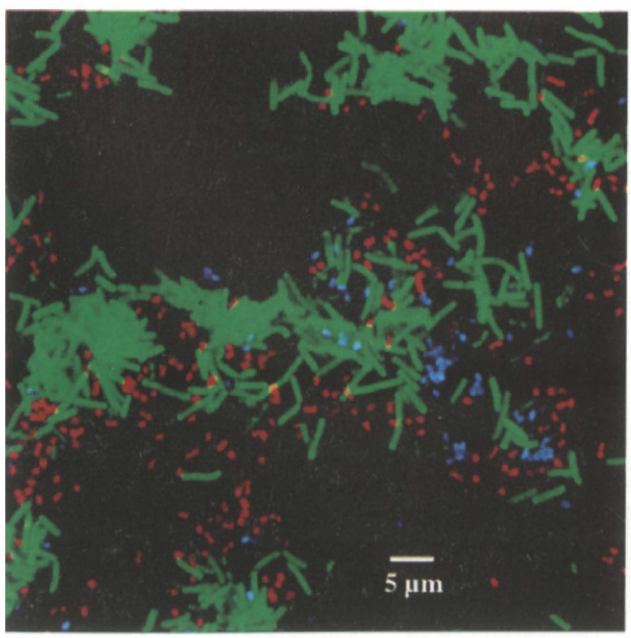
EUB338 coverage was experimentally analyzed by separate whole cell hybridization of *Bacillus stearothermophilus*, *Parachlamydia*-like *Acanthamoeba* symbiont UWE25, *Planctomyces limnophilus*, and *Verrucomicrobium spinosum* representing bacteria which possess the full match EUB338 target site, and the above described type I, II, and III sequence variations of the target site, respectively. Probe dissociation curves were recorded for increasingly stringent hybridization and washing conditions adjusted by increasing formamide concentrations in the hybridization buffer and accordingly decreasing NaCl concentrations in the wash buffer (Figure 2A–D). As expected, hybridization of *B. stearothermophilus* (representative with unaltered EUB338 target site) with probe EUB338 resulted in intense fluorescent labeling of the cells over a broad range of formamide concentrations from 0 to 70% (v/v, Fig. 2A). Further increase of stringency resulted in significant loss of signal intensity of the cells, however even the maximum applied stringency (80% formamide in the hybridization buffer and no NaCl in the wash buffer) was not sufficient to cause dissociation of 50% of the probe (equivalent to the T_d of the probe). Hybridization of the *Parachlamydia*-like *Acanthamoeba* symbiont UWE25, which has the type I sequence variation at the *E. coli* positions 338-355, with probe EUB338 at increasingly stringent conditions showed that the single T-U mismatch caused 50% probe dissociation already at 60.5% formamide but did not hamper probe binding at lower stringency (Fig. 2B). Lower signal intensities of the *Parachlamydia*-like symbiont compared to *B. stearothermophilus* within the stringency range from 0% formamide to 50% formamide were most likely caused by a lower cellular ribosome content of the symbiont cells. In contrast to bacteria with sequence variation of type I, reference cells with sequence variations of type II and III (*P. limnophilus* and *V. spinosum*, respectively) displaying three and two mismatches to probe EUB338, respectively, did not show detectable hybridization of probe EUB338 under the different conditions applied (Fig, 2C, D). These results demonstrate that a quantification of *Bacteria* in environmental samples solely by probe EUB338 would miss most members of the phyla *Planctomycetales* and *Verrucomicrobia* as well as several green non-sulfur bacteria and members of the candidate phylum OP11.

Design of probes EUB338-II and EUB338-III

Two variations of probe EUB338 that are fully complementary to the sequence variations of type II and III, EUB338-II and EUB338-III, were designed (Table 2) to

A


Probe target site sequences
EUB338 5′ ACUCCUACGGGAGGCAGC 3′
Sequence variation type I 5′ ----------U-- 3′
Sequence variation type II (EUB338-II) 5′ --A--------U---U-- 3′
Sequence variation type III (EUB338-III) 5′ --A--------U------ 3′
Planctomyces / Pirellula / Gemmata
Cytophagales*
Chlamydiales*
Isosphaera
Verrucomicrobia
WS1*
Fibrobacter
Green sulfur
Nitrospira
Termite group I
OS-K
Marine group A
Acidobacterium/Holophaga*
OP8
TM6
High GC Gram positives
OP5
OP10
Synergistes
OP3
OP9
Flexistipes
Dictyoglomus
Cyanobacteria
Coprothermobacter
Green non-sulfur*
Thermus/Deinococcus
Spirochetes*
OP11*
Thermotogales
Low GC Gram positives*
Aquificales*
Archaea
Proteobacteria*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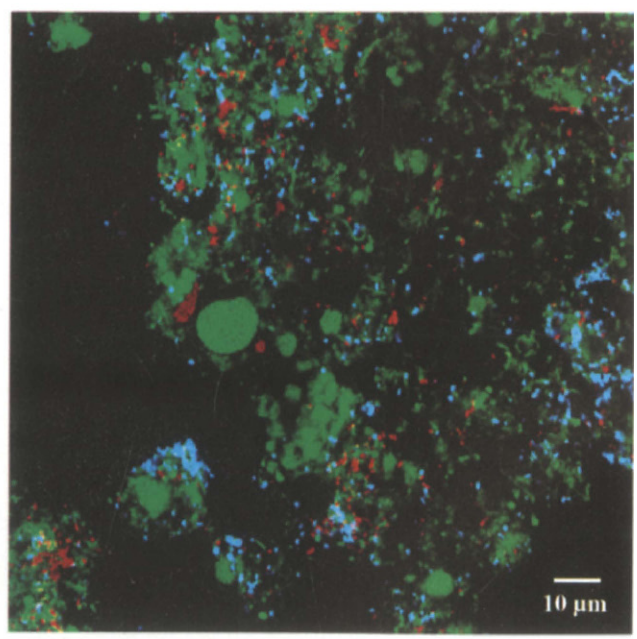
B


10 µm

C

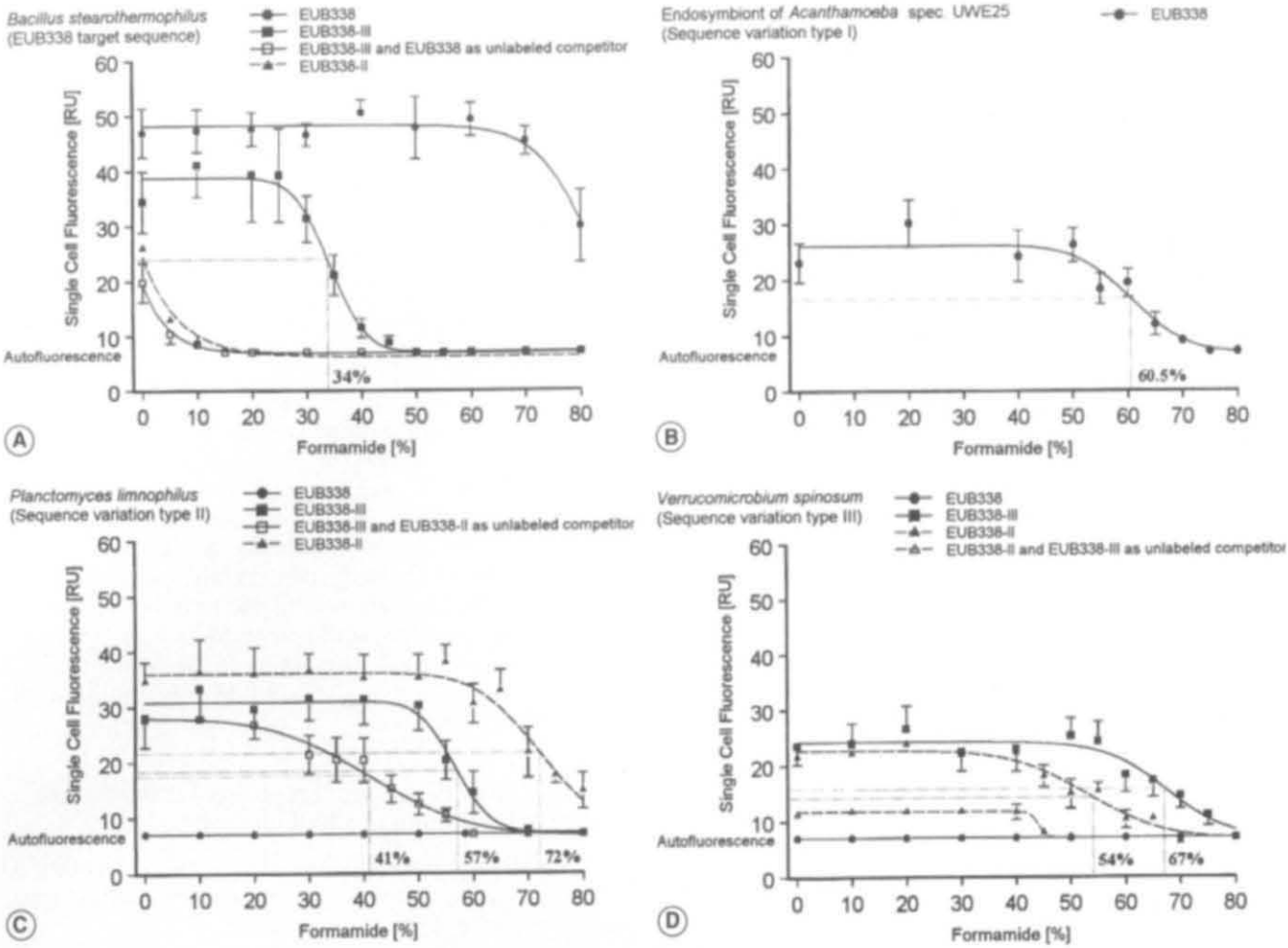


Fig. 2. Probe dissociation curves of probes EUB338, EUB338-II, and EUB338-III determined with suitable reference organisms under increasingly stringent hybridization and washing conditions. For each data point the mean fluorescence intensity of at least 100 cells was determined. Error bars indicate the standard deviation. The formamide percentage in the hybridization buffer required to cause 50% probe dissociation is given where appropriate. Organisms are (A) *Bacillus stearothermophilus* (full match to EUB338), (B) the *Parachlamydia*-like endosymbiont of *Acanthamoeba* spec. strain UWE25 (type I sequence variation), (C) *Planctomyces limnophilus* (type II sequence variation), and (D) *Verrucomicrobium spinosum* (type III sequence variation). Autofluorescence values for all reference cells under all hybridization conditions were determined to be below 10 relative units (RU).

Fig. 1. (A). Schematic representation of the phyla within the domain *Bacteria* showing the distribution of the original EUB338 target site sequence (colored green) as well as the sequence variation types I (blue), II (brown), and III (red). Phyla containing more than one sequence variation type are depicted in the resulting additive mixed color (for example the phylum *Verrucomicrobia* is labeled purple due to the presence of sequence variation type I and III, the low G+C Gram positives are colored turquoise due to the presence of the original EUB338 target site and sequence variation type I, and the candidate phylum OP11 is depicted white with brown border due to the presence of the original EUB338 target site and its three variations). The phylum *Aquificales* is given in grey with black border to illustrate that all recognized members of this lineage of descent possess additional sequence variations. Please note that the branching order of the bacterial phyla shown in this dendrogram is phylogenetically not representative.

* Additional sequence variations were observed for certain species and/or 16S rRNA sequences retrieved from environmental samples. Refer to Table 1 for details.

† Sequence variation type III was observed for the environmental clones strSMKN41 (X78664, High GC gram positives) and SBR1082 (X84513, *Cytophagales*), but since only short partial sequences are available their phylogenetic affiliation could not unambiguously be resolved.

‡ The publicly available sequences representing candidate division WS1 are incomplete and do not contain *E. coli* positions 338-355.

(B). Whole cell hybridization of an artificial mixture of *Bacillus stearothermophilus*, *Planctomyces limnophilus*, and *Verrucomicrobium spinosum* under stringent hybridization conditions with probes EUB338-FLUOS (green), EUB338-II-Cy5 (blue), and EUB338-III-Cy3 (red).

(C): *In situ* hybridization of a nitrifying biofilm from a wastewater treatment plant at Ingolstadt with probes EUB338-FLUOS (green), EUB338-II-Cy5 (blue), and EUB338-III-Cy3 (red). Some cells overlap spatially with neighbouring cells and appear therefore in mixed colors (yellow or white).

Table 2. Sequences and target sites of 16S rRNA directed oligonucleotide probes.

Probe	Sequence (5'-3')	Target site[b] (16S rRNA positions)
EUB338[a]	GCTGCCTCCCGTAGGAGT	338-355
EUB338-II	GCAGCCACCCGTAGGTGT	338-355
EUB338-III	GCTGCCACCCGTAGGTGT	338-355

[a] AMANN et al. (1990)
[b] *E. coli* numbering (BROSIUS et al., 1981)

allow for more encompassing coverage of the bacterial domain by *in situ* hybridization. Evaluation of probe specificities and optimal hybridization conditions were performed using the reference organisms *B. stearothermophilus*, *P. limnophilus*, and *V. spinosum* as representatives with 16S rRNA full-match target sites for probes EUB338, EUB338-II, and EUB338-III, respectively.

As expected, probe EUB338-II showed strong binding to the target cells of *P. limnophilus* (Fig. 2C). At 72% formamide in the hybridization buffer and without NaCl in the wash buffer probe dissociation was 50%. It should be noted, however, that the mean cellular signal intensity of the *P. limnophilus* cells after application of probe EUB338-II was, due to the lower cellular ribosome content, lower compared to the respective values of *B. stearothermophilus* with probe EUB338. Probe EUB338-II binding to *B. stearothermophilus*, which has 3 mismatches within the probe target site, was only observed at a low hybridization stringency and could be completely prevented by applying moderately stringent conditions (20% formamide in the hybridization buffer and 0.225 M NaCl in the wash buffer; Fig. 2A). In contrast, *V. spinosum* cells having only a single T-U mismatch within the probe EUB338-II target site showed binding of probe EUB338-II unless highly stringent hybridization conditions (more than 70% formamide in the hybridization buffer and no NaCl in the wash buffer) were used. Addition of equimolar amounts of unlabeled EUB338-III as competitors, however, suppressed binding of probe EUB338-II to *V. spinosum* at formamide concentrations higher than 47% in the hybridization buffer (Fig. 2D). A typical sigmoid dissociation curve was obtained after hybridization of sequence type III representative *V. spinosum* with probe EUB338-III. Half-maximal probe binding was measured at 67% formamide in the hybridization buffer and without NaCl in the wash buffer (Fig. 2D). Observed mean signal intensities for *V. spinosum* with probe EUB338-III were significantly lower compared to the values measured for *P. limnophilus* with probe EUB338-II and *B. stearothermophilus* with probe EUB338. Probe EUB338-III hybridized to *B. stearothermophilus* (two mismatches in the probe target site) under low and medium stringency conditions. After addition of an equimolar amount of probe EUB338, hybrid formation between probe EUB338-III and *B. stearothermophilus* 16S rRNA was only observed under conditions of very low stringency (Fig. 2A). Hybridization results obtained with probe EUB338-III and the *P. limnophilus* reference cells reflected the presence of the single marginal T-U mismatch in the probe target site of this species. At low and medium stringency (from 0 to 50% formamide in the hybridization buffer) probe conferred fluorescence per cell was not significantly reduced compared to hybridization with the full-match probe EUB338-II. At higher stringency the one-mismatch probe EUB338-III showed less binding than probe EUB338-II. The discrimination got more pronounced when equimolar amounts of unlabeled probe EUB338-II were added as competitors to the hybridization buffers (Fig. 2C).

Whole cell hybridization experiments were performed with artificial mixtures of *B. stearothermophilus*, *P. limnophilus* and *V. spinosum* cells and probes EUB338, EUB338-II, and EUB338-III labeled with different fluorescent dyes in order to confirm the results of the previously described formamide concentration series. As expected, specific hybridization of the reference cells with the respective probes could be achieved by applying stringent hybridization conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer; Fig. 1B).

Application of the bacterial probe set for fluorescent *in situ* hybridization of a biofilm sample

A biofilm sample originating from a continuos nitrifying reactor of a pilot wastewater treatment plant (Ingolstadt, Germany) was analyzed by simultaneous hybridization with probes EUB338, EUB338-II, and EUB338-III labeled with different fluorescent dyes under stringent conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer). Double or triple hybridization of cells was not observed under these conditions. All organisms stained by probe EUB338-II were spherical or ovoid cocci and occurred more frequently as cell clusters or chains than as single cells. The probe-conferred fluorescence was distributed in a ring-shaped manner as previously observed for cells detected *in situ* with planctomycetes-specific probes (NEEF et al., 1998). The bacteria stained by probe EUB338-III were more diverse with respect to their morphology. Cocci occurring in groups or as single cells were frequently observed (Fig. 1C). In addition, spindle-shaped cell pairs attached to each other at one pole and, to lower frequency, short rods and thin filaments were detected. Interestingly, almost all DAPI-stained cells in the sample were detectable by fluorescent *in situ* hybridization with the combination of the three probes EUB338, EUB338-II, and EUB338-III.

Discussion

Probe EUB338 is commonly used in rRNA-hybridization based molecular ecological studies in order to quantify the members of the domain *Bacteria* within complex

samples. In the *in situ* format, probe EUB338 hybridization is often applied simultaneously with DNA-binding fluorescent dyes (e.g. DAPI) to determine the bacterial fraction of total cell counts which can be visualized by *in situ* hybridization (for references see Table 3). Furthermore, probe EUB338 is frequently applied together with other probes (labeled with different fluorescent dyes) with more narrow specificities to determine the relative abundance of probe-defined populations. The encompassing database check of probe EUB338 specificity performed in this study revealed that several members of the bacterial domain do not possess a fully complementary target site of probe EUB338. Interestingly, all members of the bacterial phyla *Aquificales*, *Verrucomicrobia*, *Chlamydiales* and *Planctomycetales* show differences to the EUB338 target. Among the observed variations of the probe EUB338 target sites three sequence variation types were abundant. Sequence variation type I (present in 1.5% of the 16S rRNA database entries), which can be found within the phyla *Chlamydiales*, the Gram-positive bacteria with a low DNA G+C content, the candidate phylum OP11, and the genus *Isosphaera* is characterized by a single mismatch to probe EUB338. Whole cell hybridization experiments demonstrated that over a wide range of hybridization conditions (0–60% formamide in the hybridization buffer and 0.9–0.008 M NaCl in the wash buffer) hybrid destabilization due to this mismatch is too weak to hamper probe EUB338 binding. Thus, bacteria whose 16S rRNA molecules possess this sequence variation will still be targeted by probe EUB338 under the given hybridization conditions. However, additional hybridization experiments demonstrated that all bacteria characterized by sequence variation types II and III (found in 0.8% and 0.7% of the 16S rRNA database entries, respectively) cannot be detected *in situ* by using probe EUB338. Sequence variation types II and III are present within the candidate phylum OP11, the green non-sulfur bacteria, the *Verrucomicrobia*, and the *Planctomycetales*. Recent molecular analysis demonstrated a previously not recognized importance of these bacterial phyla for many ecosystems. rRNA sequences of the candidate phylum OP11, for which no cultured members are available, were successfully amplified from a Yellowstone hot spring (HUGENHOLTZ et al., 1998b), Carolina Bay sediment (WISE et al., 1997), and Amazonian soil (BORNEMAN and TRIPLETT, 1997). Recent unpublished data discussed by HUGENHOLTZ et al. (1998a) indicate OP11 occurrence in hydrocarbon-contaminated soils and deep-subsurface water, too. Application of the full cycle rRNA approach revealed high diversity and significant abundance of *Herpetosiphon*-like bacteria (members of the green non-sulfur bacteria) in an industrial activated sludge (JURETSCHKO et al., in preparation). Of particular note are the *Planctomycetales* and the *Verrucomicrobia* which have been shown to be more widespread in nature than initially expected. All described species of the *Planctomycetales* are freshwater or marine bacteria (e.g. HIRSCH and MÜLLER (1985), SCHLESNER (1986)), but meanwhile members of this phylum have been detected in activated sludge (BOND et al., 1995; SNAIDR et al., 1997; NEEF et al., 1998, JURETSCHKO et al, in preparation) and even in terrestrial habitats (LIESACK and STACKEBRANDT, 1992; ZARDA et al., 1997). The *Verrucomicrobia* seem to be similarly widespread in nature as they occur not only

Table 3. Studies where probe EUB338 was used to quantify bacteria in environmental samples and where the amount of cells or rRNA detected by EUB338 was compared to the total cell number or the total amount of extracted rRNA, respectively.

Publication	Samples or habitat examined	Total cell or rRNA detection method	percentage of cells or rRNA detected by EUB338
ALFREIDER et al., 1996	winter cover and pelagic layers of high mountain lake	DAPI	70–85%
FRY et al., 1997	anaerobic, alkaline aquifers	quantitative dot blot with universal probe	64–92%
GLÖCKNER et al., 1996	planctonic cells from oligo- and mesotrophic lakes	DAPI	29–64%
HICKS et al., 1992	bacterioplancton from artificial ponds	DAPI	35–67%
KALMBACH et al., 1997	drinking water biofilms	DAPI	80–90%
KÄMPFER et al., 1996	activated sludge	DAPI	70–82%
LLOBET-BROSSA et al., 1998	marine sediments	DAPI	30–73%
MANZ et al., 1994	activated sludge	DAPI	74–89%
MANZ et al., 1993	drinking water biofilms and free-water-phase	DAPI	40–70%
NEEF et al., 1996	biofilms from denitrifying sand filters	DAPI	40–80%
PERNTHALER et al., 1998	limnic picoplancton and filaments	DAPI	26–100%
RAMSING et al., 1996	water column of Mariager Fjord, Denmark	ethidium bromide staining	50%
RAMSING et al., 1996	water column of Golfo Duce, Costa Rica	ethidium bromide staining	10–20%
SNAIDR et al., 1997	activated sludge	DAPI	81%
STOFFELS et al., 1998	wastewater, biofilm	DAPI	68–90%
WAGNER et al., 1994	activated sludge	DAPI	78–83%
WAGNER et al., 1993	activated sludge	DAPI	70–90%
WALLNER et al., 1995	activated sludge	Hoechst 33342 staining	70–80%
WEISS et al., 1996	limnetic, organic aggregates	DAPI	55–100%

in aquatic environments (STALEY et al., 1976; SCHLESNER, 1987; HIORNS et al., 1997; ZWART et al., 1998) but also in sediments (WISE et al., 1997) and soils (LIESACK and STACKEBRANDT, 1992; JANSSEN et al., 1997; FELSKE et al., 1998). Moreover, FELSKE and AKKERMANS (1998) reported that a member of this phylum might be among the most active metabolizers in a terrestrial habitat.

Past research performed in various ecosystems has consistently demonstrated that a significant fraction of total cells was missed by *in situ* or dot blot hybridization with probe EUB338 (Table 3). By FISH this fraction could rarely be assigned to the domains *Archaea* or *Eucarya* by probes for those domains and therefore it was speculated that bacterial cells with a low ribosome content or impermeable cell peripheries are responsible for this (AMANN et al., 1995). It was indeed shown that specific cell fixation and permeabilization protocols (MANZ et al., 1994; ERHART et al., 1997; SCHUPPLER et al., 1998) may significantly increase the fraction of detected cells. Keeping in mind the potential environmental significance of bacteria with sequence variation types II and III it appears to be likely that these bacteria also contribute to the observed differences between EUB338 and total cell counts. Consequently, we designed the supplementary probes EUB338-II and EUB338-III which are fully complementary to the sequence variation types II and III. Optimal hybridization conditions were determined for both probes by whole cell hybridization of suitable reference strains. When used simultaneously under stringent conditions (60% formamide in the hybridization buffer and 0.008 M NaCl in the wash buffer) all three EUB338 probes specifically bound to their respective target sites. In a biofilm sample from a sewage treatment plant, nearly all DAPI-stained cells were detected by *in situ* hybridization when the three EUB338 probes were applied in combination. In the future, it should be tested whether a 1:1:1 mixture of Cy3-labeled probes EUB338, EUB338-II and EUB338-III also detects significantly higher fractions of DAPI-stained cells in other environments. If the three probes are applied with different fluorescent dyes, additional information about abundance and distribution of the little-known bacterial phyla *Planctomycetales*, *Verrucomicrobia*, green-non sulfur bacteria, and OP11 in nature can be obtained. It should, however, be stressed, that other sequence variations of probe EUB338 target site than those analyzed in this study do exist in a considerable number of bacteria (Table 1). Keeping in mind our limited knowledge of the extend of microbial diversity it is possible or even likely (i) that sequence variation types II and III are also present in novel yet not known bacterial phyla, and (ii) that yet not known bacteria with additional variations do exist in nature. Consequently, the coverage of the bacterial probes, like for all other rRNA-targeted probes, has to be continuously reevaluated and probe modifications or new probes have to be designed if necessary.

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