***In situ* probing of Gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides**

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23S-rRNA-targeted oligonucleotide probes were designed for the phylogenetic group 'Gram-positive bacteria with high G + C content of DNA' (GPBHGC). A sequence idiosyncrasy in two adjacent base pairs in the stem of helix 69 in domain IV of the 23S rRNA is present in all hitherto analysed strains of GPBHGC. An oligonucleotide probe targeted to this region hybridized only with strains of GPBHGC and was successfully used for *in situ* monitoring of these cells in activated sludge. Another unique feature of the 23S rRNA molecules of GPBHGC is a large insertion in domain III. Fluorescent oligonucleotides targeted to the highly variable regions of the rRNA within the insertions of *Corynebacterium glutamicum* DSM 20300^T, *Aureobacterium testaceum* DSM 20166 and *Brevibacterium* sp. DSM 20165 hybridized specifically to their target strains, whereas probing with oligonucleotides complementary to the rRNA-coding strand of the 23S rDNA and to the spacer between 16S and 23S rRNA of *C. glutamicum* did not result in detectable fluorescence. This confirmed that the large 23S insertions are indeed present in 23S rRNAs of GPBHGC and provide potential target sites for highly specific nucleic acid probes.

Keywords: oligonucleotide probes, 23S rRNA, *in situ* hybridization, Gram-positive bacteria, insertion

INTRODUCTION

The sequencing of rRNA molecules is not only facilitating the reconstruction of phylogenetic trees (Woese, 1987; Ludwig & Schleifer, 1994), it is also the basis for the development of oligonucleotide probes. rRNA molecules are ideal targets for nucleic acid probes for several reasons (Stahl & Amann, 1991), e.g. the primary structures of 16S and 23S rRNA are composed of sequence regions of higher and lower evolutionary conservation. Comparison of a newly retrieved rRNA sequence to the continuously growing rRNA databases allows the directed design of oligonucleotide probes complementary to more or less variable target regions. The specificity of such rRNA-targeted oligonucleotide probes can be tailored to the needs of the investigator, reaching from the subspecies (Stahl *et al.*, 1988) to the kingdom (Giovannoni *et al.*,

1988) level. Intermediate group-specific oligonucleotides complementary to sequence regions characteristic of phylogenetic entities at the genus or subclass level have been used successfully for rapid identification of bacteria (Amann *et al.*, 1990b, 1992; Devereux *et al.*, 1992; Manz *et al.*, 1992). Such probes have the potential to close further the gap between standard identification schemes and our current perception of the natural classification of bacteria.

Molecular taxonomists found significant rRNA homologies between actinomycetes and a group of related bacteria, all characterized by Gram-positive staining and a high G + C content of DNA, and consequently proposed a group named the 'actinomycetes branch' (Stackebrandt, 1991) or 'Gram-positive bacteria with high G + C content of DNA' (GPBHGC; Woese, 1987). Many species of this phylogenetic group have biotechnological or medical importance. As part of an ongoing project we intended to construct a group-specific oligonucleotide probe for this important taxon of the domain *Bacteria*. Comparative analyses of 14 complete 23S rRNA sequences of

Abbreviations: DIG, digoxigenin; GPBHGC, 'Gram-positive bacteria with high G + C content of DNA'.

Table 1. List of strains with arrangement of nucleic acids on the dot-blot membrane

Position	Organism	Source/strain*
A. 1	<i>Azospirillum amazonense</i>	DSM 2787 ^T
A. 2	<i>Azospirillum brasilense</i>	DSM 1690 ^T
A. 3	<i>Azospirillum halopraeferens</i>	DSM 3675 ^T
A. 4	<i>Rhodospirillum rubrum</i>	DSM 107
A. 5	<i>Agrobacterium tumefaciens</i>	ATCC 23308 ^T
A. 6	<i>Bradyrhizobium japonicum</i>	DSM 30131 ^T
A. 7	<i>Magnetospirillum gryphiswaldense</i>	DSM 6361 ^T
A. 8	<i>Rhizobium meliloti</i>	DSM 30135 ^T
A. 9	<i>Rhodopseudomonas palustris</i>	DSM 123 ^T
A. 10	<i>Brevundimonas diminuta</i>	DSM 1635
A. 11	<i>Paracoccus denitrificans</i>	DSM 65 ^T
A. 12	<i>Rhodobacter capsulatus</i>	DSM 1710 ^T
B. 1	<i>Thiobacillus acidophilus</i>	DSM 700 ^T
B. 2	<i>Zoogloea ramigera</i>	WS 1610
B. 3	<i>Comamonas testosteroni</i>	DSM 50244 ^T
B. 4	<i>Sphaerotilus natans</i>	DSM 565
B. 5	<i>Aquaspirillum metamorphum</i>	DSM 1837 ^T
B. 6	<i>Alcaligenes eutrophus</i>	DSM 531 ^T
B. 7	<i>Alcaligenes faecalis</i>	ATCC 8750 ^T
B. 8	<i>Chromobacterium violaceum</i>	DSM 30191 ^T
B. 9	<i>Burkholderia cepacia</i>	DSM 50181
B. 10	<i>Acinetobacter calcoaceticus</i>	LMG 1046 ^T
B. 11	<i>Alteromonas putrefaciens</i>	DSM 50426
B. 12	<i>Leucothrix mucor</i>	DSM 2157 ^T
C. 1	<i>Aeromonas hydrophila</i>	WS 1406
C. 2	<i>Vibrio anguillarum</i>	NCIMB 2129
C. 3	<i>Escherichia coli</i>	DSM 30083 ^T
C. 4	<i>Enterobacter aerogenes</i>	WS 1292
C. 5	<i>Enterobacter cloacae</i>	WS 1293
C. 6	<i>Erwinia carotovora</i>	WS 1394
C. 7	<i>Proteus vulgaris</i>	WS 1356
C. 8	<i>Serratia marcescens</i>	WS 1359
C. 9	<i>Micrococcus sedentarius</i>	DSM 20317
C. 10	<i>Pseudomonas alcaligenes</i>	DSM 50342 ^T
C. 11	<i>Pseudomonas aeruginosa</i>	DSM 50071 ^T
C. 12	<i>Pseudomonas pseudoalcaligenes</i>	LMG 1225 ^T
D. 1	<i>Corynebacterium glutamicum</i>	DSM 20300 ^T
D. 2	<i>Micrococcus luteus</i>	CCM 169 ^T
D. 3	<i>Rhodococcus rhodochrous</i>	DSM 43008
D. 4	<i>Brevibacterium linens</i>	DSM 20425 ^T
D. 5	<i>Brevibacterium</i> sp.	DSM 20165
D. 6	<i>Pimelobacter simplex</i>	DSM 20130 ^T
D. 7	<i>Propionibacterium freudenreichii</i>	DSM 20271 ^T
D. 8	<i>Flavobacterium ferrugineum</i>	DSM 30193 ^T
D. 9	<i>Cytophaga johnsonae</i>	GBF Cy j1
D. 10	<i>Mycococcus fulvus</i>	GBF Mx f2
D. 11	<i>Mycococcus virescens</i>	GBF Mx v4
D. 12	<i>Pseudomonas putida</i>	DSM 291 ^T
E. 1	<i>Bacillus cereus</i>	DSM 31 ^T
E. 2	<i>Bacillus subtilis</i>	ATCC 6633
E. 3	<i>Clostridium acetobutylicum</i>	NCIMB 8052 ^T
E. 4	<i>Clostridium stercorarium</i>	NCIMB 11754 ^T
E. 5	<i>Pectinatus frisingensis</i>	DSM 20465
E. 6	<i>Enterococcus faecium</i>	DSM 20477 ^T

E. 7	<i>Enterococcus faecalis</i>	DSM 20478 ^T
E. 8	<i>Lactobacillus casei</i>	LMG 9091
E. 9	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	DSM 20481 ^T
E. 10	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	DSM 20069 ^T
E. 11	<i>Staphylococcus aureus</i>	DSM 20231 ^T
E. 12	<i>Streptococcus salivarius</i>	DSM 20560 ^T
F. 1	<i>Staphylococcus carnosus</i>	DSM 20501 ^T
F. 2	<i>Hansenula anomala</i>	DSM 70255
F. 3	<i>Saccharomyces carlsbergensis</i>	TUM 66
F. 4	<i>Saccharomyces cerevisiae</i>	DSM 70449 ^T

* ATCC, American Type Culture Collection, Rockville, MD, USA; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany (Professor H. Reichenbach); LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; MPI, Max-Planck-Institut für Biochemie, Martinsried/München, Germany (Dirk Schüler); NCIMB, The National Collections of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, UK; TUM, Technischen Universität München, Institut für Brauereitechnologie und Mikrobiologie; WS, Institut für Mikrobiologie, Forschungszentrum für Milch und Lebensmittel, TU München, Freising/Weihenstephan.

GPBHG [Bifidobacterium bifidum DSM 20456, Brevibacterium linens DSM 20425^T, Cellulomonas flavigena DSM 20109^T, Corynebacterium glutamicum ASO 19, Gardnerella vaginalis DSM 4944^T, Nocardia asteroides DSM 43263, Propionibacterium freudenreichii DSM 20271^T, Rhodococcus erythropolis DSM 43066^T (Roller *et al.*, 1992), Micrococcus luteus DSM 20030^T (Regensburger *et al.*, 1988), Streptomyces ambifaciens ATCC 23877^T (Pernodet *et al.*, 1989), Streptomyces griseus subsp. griseus KCTC 9080 (Kim *et al.*, 1991), Frankia sp. (Simonet *et al.*, 1991), Mycobacterium leprae (Liesack *et al.*, 1991), Mycobacterium gastris ATCC 15754 (EMBL data library)] revealed both a potential target site for a group-specific oligonucleotide probe in helix 69 of the 23S rRNA domain IV and a hypervariable insertion between helix 54 and 55 in the 23S rRNA domain III that could serve as a target for highly specific probes.

Whole-cell hybridization with fluorescently labelled, rRNA-targeted oligonucleotides allows the identification of individual microbial cells (DeLong *et al.*, 1989; Amann *et al.*, 1990a). This rapid technique for monitoring microbial populations is not limited by the problems inherent in pure culture techniques (Wagner *et al.*, 1993) but is influenced by the abundance and accessibility of the intracellular rRNA target molecules. A correlation between growth rate, cellular rRNA content and the hybridization signal has been shown (DeLong *et al.*, 1989; Wallner *et al.*, 1993). The accessibility of intracellular target nucleic acids (in this case rRNA) is dependent on the permeability of the cell periphery (e.g. cell walls, cell membranes, capsules) to the probe molecules and the availability of the probe-binding site for hybridization. Not surprisingly, permeabilization of Gram-positive bac-

Table 2. List of strains with results of whole-cell hybridization

Species	Strain*		Reaction with probe		
			EUB	HGC	NHGC
<i>Actinomadura citrea</i>	43461 ^T	DSM	+	+	—
<i>Actinoplanes campanulata</i>	43148 ^T	DSM	+	+	ND
<i>Actinoplanes missouriensis</i>	43046 ^T	DSM	+	+	ND
<i>Arthrobacter globiformis</i>	13344	ATCC	+	+	—
<i>Arthrobacter ramosus</i>	1646	CCM	+	+	ND
<i>Aureobacterium testaceum</i>	20166	DSM	+	+	—
<i>Brevibacterium linens</i>	20425 ^T	DSM	+	+	—
<i>Brevibacterium</i> sp.	20165	DSM	+	+	—
<i>Cellulomonas cellulans</i>	20106	DSM	+	+	—
<i>Cellulomonas flavigena</i>	20109	DSM	+	+	—
<i>Corynebacterium glutamicum</i>	20300 ^T	DSM	+	+	—
<i>Frankia</i> sp.	Ag45/m15	AUW	+	+	—
<i>Frankia</i> sp.	AgB1.9	AUW	+	+	ND
<i>Microbacterium imperiale</i>	20530	DSM	+	+	—
<i>Micrococcus agilis</i>	2390 ^T	CCM	+	+	ND
<i>Micrococcus kristinae</i>	27570 ^T	ATCC	+	+	—
<i>Micrococcus luteus</i>	10240	ATCC	+	+	ND
<i>Micrococcus luteus</i>	169 ^T	CCM	+	+	—
<i>Micrococcus sedentarius</i>	20317	DSM	+	+	ND
<i>Micromonospora echinospora</i>	43141 ^T	DSM	+	+	ND
<i>Microtetraspora glauca</i>	23057 ^T	ATCC	+	+	—
<i>Nocardia asteroides</i>	43263	DSM	+	+	ND
<i>Nocardia calcarata</i>	43065	DSM	+	+	ND
<i>Pimelobacter simplex</i>	20130 ^T	DSM	+	+	—
<i>Rhodococcus erythropolis</i>	43066	DSM	+	+	ND
<i>Rhodococcus rhodochrous</i>	43241	DSM	+	+	—
<i>Streptomyces humiferus</i>	43030 ^T	DSM	+	+	—
<i>Streptomyces</i> sp.	118	TUHH	+	+	—
<i>Streptomyces</i> sp.	68	TUHH	+	+	ND
<i>Eubacterium lentum</i>	2243	DSM	+	—	+
<i>Enterococcus faecium</i>	20477 ^T	DSM	+	—	ND
<i>Streptococcus thermophilus</i>	20479 ^T	DSM	+	—	+
<i>Lactobacillus rhamnosus</i>	20024	DSM	+	—	ND
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	20481 ^T	DSM	+	—	ND
<i>Staphylococcus aureus</i>	20231 ^T	DSM	+	—	ND
<i>Bacillus cereus</i>	31 ^T	TUM	+	—	+
<i>Brevundimonas diminuta</i>	1635	DSM	+	—	+
<i>Comamonas testosteroni</i>	50244 ^T	DSM	+	—	+
<i>Escherichia coli</i>	30083 ^T	DSM	+	—	+
<i>Cytophaga johnsonae</i>	Cy j1	GBF	+	—	+
<i>Myxococcus virescens</i>	Mx v4	GBF	+	—	+

ND, Not done.

* TUHH, Technischen Universität Hamburg-Harburg (Brigitte Böckle); AUW, Agricultural University Wageningen (Dr. Dittmar Hahn). See Table 1 for other abbreviations.

teria with their rigid cell walls has been more difficult than permeabilization of Gram-negative bacteria. Different protocols have been developed for Gram-positive bacteria: (i) fixation in 3 % (w/v) paraformaldehyde solution followed by a partial enzymic digestion of the cell wall (Hahn *et al.*, 1992; Beimfohr *et al.*, 1993); (ii) air-drying followed by heat-fixation (Jurtshuk *et al.*, 1992); and (iii)

fixation in an alcohol (methanol or ethanol)/formaldehyde mixture (DeLong *et al.*, 1989; Braun-Howland *et al.*, 1992). Another effect, the unavailability of specific rRNA target sites in permeabilized fixed cells, has only been reported recently (Amann *et al.*, 1992) and is probably due to the higher order structure of the ribosome or rRNA.

Table 3. Probe sequences and target sites

Probe	Sequence	Target organism(s)	Target site
HGC	5'-TATAGTTACCTCCGCCGT-3'	GPBHGC	1901–1918*
NHGC	5'-TATAGTTACGGCCGCCGT-3'	<i>Bacteria</i> , not GPBHGC	1901–1918*
ATE	5'-GAAGGGATCCGCTAAAGC-3'	<i>A. testaceum</i>	46–63†
BKE	5'-GGTTAGCATCACTGATTCA-3'	<i>Brevibacterium</i> sp.	12–30†
CGL	5'-TTATGGGTGGTTAGTATC-3'	<i>C. glutamicum</i>	23–40†
aCGL	5'-GATACTAACCACCCATAA-3'	<i>C. glutamicum</i>	23S rDNA†
CGLSF	5'-ACTTTGTGTGTGGG-3'	<i>C. glutamicum</i>	Spacer 16S–23S, rDNA
CGLSR	5'-CCCACACACAAAGT-3'	<i>C. glutamicum</i>	Spacer 16S–23S, rDNA

* rRNA position, *E. coli* numbering (Brosius *et al.*, 1981).

† Insertion between helix 54a and 55a (Roller *et al.*, 1992).

Fig. 1. Secondary structure model of part of domain IV of bacterial 23S rRNA. The consensus sequence shown is based on a data set of about 100 complete 23S rRNA sequences from representatives of all bacterial phyla. Invariant residues are indicated by upper-case letters, those which are invariant in at least 90% of the sequences are indicated as lower-case letters and less conserved positions are represented by circles. The target site of probes HGC and NHGC is visualized by bold italics. Arrowheads highlight the signature positions. Base numbering is according to Brosius *et al.* (1981); helix numbering is according to Höpfel *et al.* (1989).

In this study, cell fixation and hybridization parameters were optimized for whole cell identification of GPBHGC,

and probes directed against characteristic sequence regions – so-called signatures – on the 23S rRNA were evaluated.

METHODS

Organisms and culture conditions. Sources and strain numbers of the organisms investigated in this study are listed in Tables 1 and 2. Cells were cultured as described in the catalogues of strains of the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), ATCC (American Type Culture Collection, Rockville, MD, USA) or CCM (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia) or were kindly provided by various investigators (see Tables 1 and 2).

Cell fixation. Cells growing in exponential phase (OD_{600} between 0.5 and 0.8) were harvested by centrifugation (2 min, 5000 *g*) and washed in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4). Gram-positive bacteria were resuspended in PBS and fixed by addition of ethanol (50%, v/v). Activated sludge was fixed at the time of sampling by addition of ethanol to a final concentration of 50%. Gram-negative bacteria were fixed for 3 h in 3% paraformaldehyde/PBS, washed in PBS and finally resuspended (Amann *et al.*, 1990b). All cells were stored in PBS/96% ethanol (1:1, v/v) at -20°C .

Oligonucleotide probes. All probe sequences are given in Table 3. Oligonucleotide probes were synthesized with a C6-TFA aminolinker [6-(trifluoroacetyl amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite] at the 5'-end (MWG Biotech). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes) and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim) and purification of the oligonucleotide-dye conjugates was performed as described by Amann *et al.* (1990a). Probes were also labelled with digoxigenin (DIG) following the protocols of Zarda *et al.* (1991).

Whole-cell hybridization. Fixed cells were spotted on pre-cleaned, gelatin-coated [0.1% gelatin, 0.01% $\text{KCr}(\text{SO}_4)_2$] microscopic slides (Paul Marienfeld KG, Bad Mergentheim, Germany), and immobilized by drying at 46°C for 15 min. A series of immersions in 50%, 80% and 96% (v/v) ethanol

Fig. 2. Potential secondary structure models of the large stable insertions in domain III (Roller *et al.*, 1992) of the 23S rRNA molecules of *A. testaceum*, *Brevibacterium* sp. and *C. glutamicum*. Target sites of probes ATE (targeting *A. testaceum*), BKE (*Brevibacterium* sp.) and CGL (*C. glutamicum*) are highlighted by bold italics.

(3 min each) completed the fixation. Samples of 8 µl hybridization solution (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01 % SDS, 50 ng probe, X % formamide) were applied to each well of the microscopic slide and incubated for 3–4 h in an isotonicity equilibrated humidity chamber. The probe was removed from the slide by rinsing with 2 ml prewarmed washing solution (20 mM Tris/HCl, 0.01 % SDS, 5 mM EDTA, Y M NaCl; the salt concentration, Y, was adjusted to the formamide concentration, X, in the hybridization buffer as described by Manz *et al.*, 1992). Slides were rapidly transferred into washing solution and incubated at 48 °C for 20 min. Slides were rinsed briefly with distilled water, air-dried and mounted in Citifluor solution.

Slides were examined using a Zeiss Axioplan microscope fitted for epifluorescence microscopy with a 50 W mercury high-pressure bulb and Zeiss filter sets nos 09 and 15. Black-and-white photomicrographs were done on Kodak Tmax400. Exposure times were 0.06 s for phase contrast and 15–30 s for epifluorescence micrographs.

Quantification of probe-conferred fluorescence. To determine the optimal hybridization stringency the signals conferred by the fluorescein-labelled oligonucleotide probe HGC, specific for GPBHGC, was determined at different formamide concentrations in the hybridization buffer by image analysis according to Trebesius *et al.* (1994). Formamide can be used to influence the sensitivity and specificity of hybridization (Stahl & Amann, 1991).

Dot-blot analysis. Oligonucleotide probe specificity was examined by hybridization of DIG-labelled probes to reference nucleic acids extracted from 64 pure cultures of bacteria and eukarya representing a diverse collection of taxa. Reference

nucleic acids were isolated using a modification of the method of Oelmüller *et al.* (1990). Cells (usually 2 ml cell suspension) were harvested by centrifugation (10 min; 18000 g; 4 °C) in a Hettich centrifuge 2021, washed with acetate/EDTA (AE) buffer (20 mM sodium acetate, 1 mM disodium EDTA, pH 5.5; 0 °C) and resuspended in a mixture of 400 µl AE buffer and 50 µl 25 % (w/v) SDS. Gram-positive cells and yeast cells were mechanically disrupted using glass beads (0.17 mm diameter) in combination with sonication (2 min). Nucleic acids of all cells were purified by phenol extraction followed by ethanol precipitation (Manz *et al.*, 1992). The concentration of nucleic acids was determined spectrophotometrically by measuring the A_{260} . Isolated reference nucleic acids were immobilized on nylon membranes (Diagen) using a dot-blot apparatus (Schleicher and Schuell). Probing with DIG-labelled oligonucleotides was done as described previously (Manz *et al.*, 1992).

RESULTS AND DISCUSSION

Design of oligonucleotide probes

The longest coherent structure of highly conserved residues within the primary structure of bacterial 23S rRNAs comprises 83 bases of domain IV (Höpfel *et al.*, 1989; Larsen, 1992; Ludwig & Schleifer, 1994; positions 1889–1971, *Escherichia coli* numbering, Brosius *et al.*, 1981). A potential secondary structure model of part of domain IV (helices 68 and 69; Fig. 1) is shown for a consensus sequence derived from a data set of complete 23S rRNA sequences from representatives of all bacterial phyla (Woese, 1987). Within the stem of helix 69 most bacterial sequences contain CC in positions 1908–1909 (*E.*

Fig. 3. Dot-blot analysis of probe specificities. Nucleic acids were isolated from 64 reference organisms (see Table 2 for list of strains and arrangement). Membranes were hybridized from top to bottom with probes HGC, NHGC, CGL and EUB.

coli numbering), presumably paired with GG (1912–1913). However, all 23S rRNA sequences so far determined for GPBHGC have AG/CU (1908–1909/1912–1913) in place of CC/GG. In the construction of a probe best exploiting this sequence idiosyncrasy, probe HGC was designed as an 18mer complementary to positions 1901–1918. Another 18mer also binding at positions 1901–1918 and complementary to the homologous region in the 23S rRNA of all bacteria other than GPBHGC was designated NHGC. It has recently been demonstrated that mismatch discrimination can be enhanced by competitive hybridization with two oligonucleotides targeted to homologous regions (Manz *et al.*, 1992). Therefore, the combined use of probes HGC and NHGC can be of advantage for the specificity of the assay.

The presence of large stable insertions (about 100 bases)

within domain III of the 23S rRNA has been shown to be a signature for the GPBHGC (Roller *et al.*, 1992). The primary structures of the insertions are evolutionarily only moderately conserved and thus provide potential target sites for species-specific probes. Based on partial 23S rRNA sequences of *Aureobacterium testaceum*, *Brevibacterium* sp. (formerly *B. ketoglutamicum*) and *C. glutamicum*, probes ATE, BKE and CGL (Roller *et al.*, 1992) were designed complementary to parts of the large insertion between helices 54 and 55. These probes target different regions (see Table 1 and Fig. 2) within this insertion and were used to test for the presence and accessibility of their target sequences on mature 23S rRNA molecules. All of the following oligonucleotides served as controls during whole cell hybridization: aCGL is complementary to CGL and is a control for non-specific binding and for binding to the rRNA genes (rDNA) of *C. glutamicum*; CGLSR is targeted at the 16S–23S spacer on the precursor-like strand on the rRNA operon and the precursor of *C. glutamicum*, whereas CGLSF is targeted to the precursor-coding strand of the rRNA operon. Probe EUB is complementary to a region of the 16S rRNA conserved for *Bacteria* (Amann *et al.*, 1990b) and was used as positive control for the permeability of the fixed Gram-positive cells to rRNA-targeted oligonucleotides. All probe sequences are given in Table 3.

Fixation of GPBHGC for whole-cell hybridization

Initial experiments were performed with exponentially growing cells of *C. glutamicum*, *A. testaceum*, and *Brevibacterium* sp. that had been fixed in 3% paraformaldehyde for several hours and stored in ethanol/PBS (1:1, v/v) (Amann *et al.*, 1990b). Whole-cell hybridization with fluorescent derivatives of the bacterial probe EUB or the specific probes did not result in reliable visualization of target cells (data not shown). Since cells were taken from cultures growing at short generation times (approx. 1 h) cellular ribosome contents were probably high enough for detection, and the failure in detection was most likely due to the limited permeabilities of the fixed cells to probes. Treatment of paraformaldehyde-fixed cells with lysozyme has been shown to increase the permeability of Gram-positive streptococci (Beimfohr *et al.*, 1993) and *Streptomyces scabies* hyphae (Hahn *et al.*, 1992), but it did not significantly influence hybridization signals of *C. glutamicum*, *A. testaceum* and *Brevibacterium* sp. However, fixation in 50% ethanol yielded cell preparations that showed homogeneous, strong hybridization with fluorescently labelled probes (Fig. 4). This supports recently published results that paraformaldehyde fixation is not required (Jurtshuk *et al.*, 1992) and is even detrimental (Braun-Howland *et al.*, 1992) to whole cell hybridization of Gram-positive bacteria.

Determination of optimal hybridization conditions

By gradually increasing the formamide concentration at a fixed incubation temperature the hybridization stringency was optimized for each probe to achieve an optimal

Fig. 4. Whole-cell hybridization. Panel (a) Cells of *Streptococcus thermophilus* (coccoid rods), *C. glutamicum* (irregular rods) and *Microbacterium imperiale* (thin rods) were hybridized with fluorescein-labelled probe EUB and tetramethylrhodamine-labelled probe HGC. Identical fields were viewed with phase contrast (left) and epifluorescence microscopy using a fluorescein- (centre) and rhodamine-specific filter set. All three cell types have bound bacterial probe EUB; only cells of *M. imperiale* and *C. glutamicum* have bound probe HGC. Panel (b) Cells of *C. glutamicum*, *Brevibacterium* sp. (long, irregular rods) and *Pseudomonas diminuta* (thin rods) were hybridized with fluorescein-labelled probe NHGC and tetramethylrhodamine-labelled probe HGC. After hybridization *P. diminuta* cells were visualized using

balance between specificity and sensitivity. The average fluorescence signals were quantified by digital image microscopy (data not shown). At formamide concentrations of 25% (v/v) for probes HGC and NHGC and 0% for CGL, ATE and BKE the probes distinguished clearly between target and non-target cells.

Evaluation of probe specificities

Under optimal hybridization conditions, the specificities of probes HGC (complementary of the GPBHGC), NHGC (complementary to all other bacteria), CGL (complementary to *C. glutamicum*), ATE (complementary to *A. testaceum*; data not shown) and BKE (complementary to *Brevibacterium* sp.; data not shown) were experimentally evaluated in two ways.

DIG-labelled probes were used for dot-blot hybridization against nucleic acids extracted from 64 reference strains (Table 1). These reference strains were selected to cover the important phylogenetic groups within the domain *Bacteria* and included eight GPBHGC. Probes HGC and NHGC showed the expected specificities (Fig. 3, panels a and b). As expected probe CGL only hybridized to nucleic acids extracted from *C. glutamicum* (Fig. 3, panel c). Hybridization with the bacterial probe EUB (Fig. 3, panel d) served as positive control.

Whole-cell hybridizations with fluorescent derivatives of probes HGC, NHGC and EUB were performed on defined mixtures of target and non-target cells. The results are in accordance with the known phylogenetic affiliations of the hybridized cells (Table 2). Representative photomicrographs are shown in Fig. 4, panels (a) and (b).

Whole-cell hybridization to the large 23S rRNA insertion of GPBHGC

Using the optimized fixation protocol, cells of *C. glutamicum*, *A. testaceum* and *Brevibacterium* sp. could be specifically visualized with the fluorescently mono-labelled probes CGL (Fig. 4, panel c), ATE and BKE (not shown), respectively. These results indicated accessibility of the probes to the target regions and their occurrence in relatively high numbers per cell. Control hybridizations with fluorescently labelled probe CGLSR targeted to the 16S–23S spacer (present in the precursor and excised in mature rRNA) and probes aCGL and CGLSF, complementary to CGL and CGLSR, did not result in detectable probe-conferred fluorescence. These data corroborate earlier findings obtained by Southern and Northern hybridization and sequencing that the large 23S rDNA insertion of GPBHGC is not removed during the maturation of precursor rRNA (Roller *et al.*, 1992), in

Fig. 5. *In situ* detection of filamentous bacteria in activated sludge with fluorescein-labelled probe HGC. Phase-contrast (upper panel) and epifluorescence micrographs (lower panel) are shown for an identical microscopic field.

contrast to the 23S rDNA insertions in other bacteria (e.g. Burgin *et al.*, 1990; Skurnik & Toivanen, 1991). It also demonstrates that fluorescent oligonucleotides can be tools to rapidly prove the presence of insertions in mature rRNA molecules and to distinguish these stable insertions from those which are removed during rRNA processing.

In situ hybridization with probe HGC

Fluorescently labelled, group-specific oligonucleotide probes have been successfully used for *in situ* monitoring of population changes in complex microbial communities such as sulfidogenic biofilms (Amann *et al.*, 1992) or activated sludge (Wagner *et al.*, 1993). Rapid and reliable monitoring of GPBHGC in activated sludge would be beneficial since these bacteria have been connected with malfunctions such as foaming or bulking (Blackall *et al.*, 1989, 1991; Lemmer & Kroppenstedt, 1984). Hybridization of activated-sludge samples with probe HGC resulted in the visualization of specific morphotypes, e.g. filamentous bacteria (Fig. 5). There was a marked influence of sample fixation on the number of cells

the fluorescein-specific filter set (centre) and cells of *C. glutamicum* and *Brevibacterium* sp. were visualized using the rhodamine-specific filter set. Panel (C) Tetramethylrhodamine-labelled probe CGL allows specific detection of *C. glutamicum* in a mixture of *S. thermophilus*, *C. glutamicum* and *M. imperiale*. Phase-contrast (left) and epifluorescence (right) micrographs are shown for an identical field.

detected by probe HGC. When the sample had been fixed in paraformaldehyde only $10.7 \pm 3.5\%$ of the cells detected with the DNA-intercalating dye 4',6-diamidino-2-phenylindole (DAPI) were visualized by probe HGC, but, when the sample had been fixed in ethanol, $22.9 \pm 6.6\%$ hybridized with probe HGC. This clearly showed the importance of adequate sample preparation for quantitative monitoring of groups by *in situ* probing (Manz *et al.*, 1994).

Gram-staining was positive for most of the morphologically distinct cell types detected by probe HGC, but proved difficult to interpret in activated-sludge samples. Thus, for the *in situ* monitoring of GPBHGC, fluorescent oligonucleotide probing could become a valuable complement to standard staining techniques and immunofluorescence. Simultaneous hybridization of probe HGC with differently labelled probes for the alpha-, beta-, and gamma-subclasses of *Proteobacteria* (Manz *et al.*, 1992) and another group-specific probe detecting members of the genera *Cytophaga*, *Flavobacterium* and *Flexibacterium*, currently under development, did in no case result in dual staining of cells, indicating that these probes indeed detect separate groups. This is important since the diversity of complex microbial communities (such as activated sludge) is today only partially known. Therefore even an extensive evaluation of databases and reference strains cannot guarantee the intended specificity of probes.

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