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Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment

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We designed a panel of four 16S rRNA-targeted oligonucleotide probes specific for bacteria of the phylum cytophaga-flavobacter-bacteroides (CFB). Probes CF319a and CF319b are targeted to members of the flavobacteria-cytophaga group and the genus *Porphyromonas*, whereas probe BAC303 has a target region characteristic for the genera *Prevotella* and *Bacteroides* within the bacteroides group. The probe FFE8b was developed for species-specific hybridizations with *Flavobacterium ferrugineum*. All probes were designed by computer-assisted sequence analysis and compared to all currently accessible 16S and 23S rRNA sequences. The oligonucleotides were further evaluated by whole-cell and non-radioactive dot-blot hybridization against reference strains of the CFB phylum and other major lineages of *Bacteria*. The newly developed probes were used together with other higher-order probes to analyse the structure and community composition in complex environments. In activated sludge samples, members of the flavobacteria-cytophaga group were revealed by *in situ* hybridization as important constituents of sludge flocs and characteristic colonizers of filamentous bacteria. By application of fluorescent probe BAC303, members of the genera *Bacteroides* and *Prevotella* could be visualized without prior cultivation as an important part of the human faecal microflora.

Keywords: 16S rRNA-targeted oligonucleotide probes, *in situ* hybridization, phylum cytophaga-flavobacter-bacteroides, flavobacteria-cytophaga group, bacteroides group

INTRODUCTION

The phylum cytophaga-flavobacter-bacteroides (CFB) is one of the major lineages (phyla) of *Bacteria* (Woese, 1987). Members of the flavobacteria-cytophaga group within the CFB phylum are frequently isolated from many natural and man-made ecosystems (soil, fresh and marine waters, clinical specimens, air-conditioning, sewage-treatment plants) and exhibit a broad range of phenotypic diversity. By their ability to degrade macromolecules such as cellulose, agar and chitin they are of considerable practical importance. The Gram-negative, anaerobic *Bacteroides* species that form the second major group within the CFB phylum account for approximately 30% of all faecal isolates from the normal human colon microflora (Moore & Holdeman, 1974; Holdeman *et al.*, 1976). They are also the most important anaerobic bacteria associated with human infections (Salyers, 1984).

Despite the known importance of the CFB phylum, the lack of characteristic bacterial morphologies and physiologies has hindered the establishment of a valid system of classification for this phenotypically complex taxon. Since the first description of the genus *Cytophaga* (Winogradsky, 1929) and the 'colour genus', *Flavobacterium* (Bergey *et al.*, 1923) it has proven difficult to define valid criteria for the phenotypic differentiation of the genera *Flavobacterium* and *Cytophaga*. Several chemotaxonomic approaches based on pigmentation (Reichenbach *et al.*, 1981), respiratory

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Abbreviation: CFB, cytophaga-flavobacter-bacteroides.

quinone systems (Mannheim, 1981; Reichenbach, 1989) and cellular fatty acid composition (Oyaizu & Komagata, 1981) showed many kinds of relationship between the genera *Flavobacterium* and *Cytophaga*, but could not provide a reliable basis for classification of this taxon.

Nowadays the comparative analysis of phylogenetic marker molecules which provide a large set of definable criteria at the molecular and genotypic level (Ludwig & Schleifer, 1994) allows the valid phylogenetic affiliation of prokaryotes. Molecular approaches using DNA–DNA (Callies & Mannheim, 1980) and DNA–rRNA hybridizations (Bauwens & de Ley, 1981), studies on the basis of oligonucleotide cataloguing (Fox *et al.*, 1977) and comparisons of 16S rRNA sequences have revealed the natural relationships within the CFB phylum (Gherna & Woese, 1992) and between these organisms and bacteria from other taxa (Woese *et al.*, 1990a, b).

Analysis of specific signature regions within the 16S rRNA has provided more detailed insights into the relationships between members of the CFB phylum. The two primary groups, bacteroides and flavobacteria-cytophaga, have been subdivided by Gherna & Woese (1992) into five major phylogenetic subgroups: bacteroides, cytophaga, flavobacter, sphingobacter and saprospira; the latter four subgroups constitute the flavobacteria-cytophaga group. Paster *et al.* (1994) divided the bacteroides (sub)group into three clusters, named by the genera *Prevotella*, *Bacteroides* and *Porphyromonas*. The species *Flavobacterium ferrugineum* has been removed from the genus *Flavobacterium* (Holmes *et al.*, 1984) and an outlying taxonomic status has been accorded to this organism (Gherna & Woese, 1992).

Signature regions of the 16S rRNA also provide potential target sites for rRNA-targeted oligonucleotide hybridization probes (Giovannoni *et al.*, 1988; Schleifer *et al.*, 1993). Such probes can be designed on all taxonomic levels ranging from domains (Amann *et al.*, 1990a; Stahl & Amann, 1991) and other higher taxa (Manz *et al.*, 1992; Burggraf *et al.*, 1994; Roller *et al.*, 1994) to the species and subspecies level (Amann *et al.*, 1990b; Ehrmann *et al.*, 1992). Fluorescently labelled probes have been successfully applied for identification of hitherto uncultured bacteria (Amann *et al.*, 1991; Spring *et al.*, 1992) and for elucidating the community structure of diverse microbial consortia (Amann *et al.*, 1992; Wagner *et al.*, 1993; Manz *et al.*, 1993, 1994).

The aim of this work was the development of higher-order probes for the specific characterization of bacteria belonging to the CFB phylum and, in view of its outlying taxonomic status, for *Flavobacterium ferrugineum*. These probes are important parts of a comprehensive panel of rRNA-targeted oligonucleotide probes intended to facilitate the rapid analysis of microbial community structures by *in situ* hybridizations. Fluorescent derivatives of the newly developed probes specific for the flavobacteria-cytophaga group were successfully used in *in situ* hybridization experiments to examine the occurrence and spatial arrangement of these organisms within activated sludge flocs. One of the probes was used for identification and characterization of members of the genera *Bacteroides* and *Prevotella* in the human faecal flora without prior anaerobic cultivation.

METHODS

Organisms and culture conditions. Strain numbers and sources of bacteria belonging to the CFB phylum investigated in this study are listed in Table 1. Strains were cultured in media and under culture conditions as recommended in the corresponding catalogues of the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG), the GBF (Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG) and the LMG (Laboratorium voor Microbiologie, Universiteit Gent, Belgium).

Cell fixation. Cells in the exponential phase were harvested by centrifugation (2 min, 5000 ***g***) and carefully suspended in 1 ml sterile phosphate-buffered saline at pH 7·4 (PBS: 130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4). Pure cultures and activated sludge samples were fixed for at least 3 h by addition of 3 vols 4 % (w/v) paraformaldehyde at 4 °C. After washing once with PBS, cells were stored in a 1:1 mixture of PBS and 96 % (v/v) ethanol at −20 °C. Fixed cells were spotted on precleaned, gelatin-coated [0·01 % $KCr(SO_4)_2$, 0·1 % gelatin] microscope slides (Paul Marienfeld, Bad Mergentheim, FRG), air-dried and dehydrated in 50, 80, and 96 % (v/v) ethanol (3 min each).

Faecal specimens. Stool samples were obtained from two healthy adults. To obtain the bacterial fraction approximately 0·5 g faeces was suspended evenly in 1 ml sterile PBS at pH 7·4, harvested by centrifugation (2 min, 5000 ***g***), carefully resuspended and washed twice in PBS. The cell suspension was fixed for at least 3 h by addition of 3 vols 4 % (w/v) paraformaldehyde at 4 °C. After washing once with PBS, cells were stored in a 1:1 mixture of PBS and 96 % (v/v) ethanol at −20 °C. Preparation of cell smears was done as described above.

Oligonucleotide probes. An alignment of 16S rRNA sequences from 89 members of the CFB phylum and representatives of other major lineages of the *Bacteria* obtained from the Technical University Munich data collection (Dr W. Ludwig) was screened for signatures characteristic for the flavobacteria-cytophaga and the bacteroides group within the CFB phylum, as well as *Flavobacterium ferrugineum*. Currently available data sets of about 3000 complete or almost complete 16S and 23S rRNA sequences were checked by the newly developed ARB software package (Technical University Munich, Munich, FRG).

Two oligonucleotides specific for the flavobacteria-cytophaga group (probes CF319a and CF319b), one complementary to a characteristic region of the bacteroides group (probe BAC303) and one specific to 16S rRNA of *Flavobacterium ferrugineum* (probe FFE8b) were designed. Probe sequences and target positions corresponding to positions in the *Escherichia coli* 16S rRNA (Brosius *et al.*, 1981) are listed in Table 2. An alignment of probes CF319a, CF319b and BAC303 with the complementary regions of the 16S rRNAs from representative organisms of the five main subgroups of the CFB phylum and representative non-target organisms characteristic of other phyla is given in Fig. 1. Probe EUB338, complementary to a region of the 16S rRNA conserved in the domain *Bacteria* (Amann *et al.*, 1990a) was used as a positive control. Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite] at the 5′-end (MWG Biotech). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim) was performed as described pre-

Table 1. Target organisms, sources, phylogeny and results of whole-cell and dot-blot hybridizations

Organism	Source*	Phylogeny†	Hybridization signal with probe‡			
			BAC303	CF319a	CF319b	FFE8b
Bacteroides vulgatus	ATCC 8482^T	B	+	−	−	−
Bacteroides distasonis	ATCC 8503^T	B	+	+	−	−
Bacteroides eggerthii	ATCC 27754^T	B	+	−	−	−
Bacteroides fragilis	ATCC 25285^T	B	+	−	−	−
Bacteroides ovatus	ATCC 8483^T	B	+	−	−	−
Bacteroides thetaiotaomicron	ATCC 29148^T	B	+	−	−	−
Prevotella loescheii	ATCC 15930^T	B	−	−	−	−
Bergeyella zoohelcum	LMG 8351^T	CF	−	+	−	−
Bergeyella zoohelcum	LMG 8352	CF	−	−	−	−
Chryseobacterium gleum	LMG 8334	CF	−	+	−	−
Chryseobacterium indologenes	LMG 8336	CF	−	+	−	−
Chryseobacterium indologenes	LMG 8337^T	CF	−	+	−	−
Cytophaga hutchinsonii	LMG 10844^T	CF	−	−	+	−
Cytophaga johnsonae	LMG 1341^T	CF	−	+	−	−
Cytophaga johnsonae	LMG 1342	CF	−	+	−	−
Cytophaga uliginosa	LMG 3809^T	CF	−	+	−	−
Empedobacter brevis	LMG 4011^T	CF	−	+	−	−
Empedobacter brevis	LMG 4012	CF	−	+	−	−
Flavobacterium aquatile	LMG 4008^T	CF	−	+	−	−
Flavobacterium ferrugineum	LMG 4021^T	CF	−	−	−	+
Flavobacterium odoratum	LMG 1233^T	CF	−	+	−	−
Flavobacterium odoratum	LMG 4028	CF	−	+	−	−
Flexibacter columnaris	LMG 13035	CF	−	+	−	−
Flexithrix dorotheae	DSM 6795^T	CF	−	+	−	−
Haliscomenobacter hydrossis	DSM 1100^T	CF	−	+	−	−
Riemerella anatipestifer	LMG 11054^T	CF	−	+	−	−
Riemerella anatipestifer	LMG 11602	CF	−	+	−	−
Saprospira grandis	DSM 2844	CF	−	+	−	−
Sphingobacterium heparinum	LMG 4024^T	CF	−	+	−	−
Sphingobacterium mizutae	LMG 8340^T	CF	−	+	−	−
Sphingobacterium spiritivorum	LMG 8347^T	CF	−	+	−	−
Sphingobacterium spiritivorum	LMG 8348	CF	−	+	−	−
Sporocytophaga myxococcoides	LMG 8393^T	CF	−	−	+	−
Taxeobacter ocellatus	GBF Txo1	CF	−	−	−	−
Weeksella virosa	LMG 8349	CF	−	−	−	−
Weeksella virosa	LMG 8350	CF	−	−	−	−

* A superscript T denotes a type strain. Culture collections are given in full in Methods.

† B, bacteroides group; CF, flavobacteria-cytophaga group.

‡ +, Strong hybridization signal; −, no hybridization signal.

viously (Amann *et al.*, 1990b). For dot-blot hybridizations, probes were labelled with digoxigenin following the protocols of Zarda *et al.* (1991).

Nucleic acid extraction and dot-blot hybridization. For further evaluation of probe specificities whole-cell hybridizations and dot-blot analyses were performed using cells and nucleic acids of 36 different strains of organisms representing major subgroups within the CFB phylum (Table 1). The strains listed below were used as negative controls for whole-cell and dot-blot hybridizations (culture collections: ATCC, American Type Culture Collection, Rockville, MD, USA; CCM, Czechoslovak Collection of Microorganisms, Brno, CSFR; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG; GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIMB, The National Collections of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK; WS, Institut für Mikrobiologie, Forschungszentrum für Milch und Lebensmittel, Technical University Munich, Freising Weihenstephan, FRG).

α-Proteobacteria: *Agrobacterium tumefaciens* ATCC 23308; *Azospirillum amazonense* DSM 2787; *Azospirillum brasilense* DSM 1690; *Azospirillum halopraeferens* DSM 3675; *Bradyrhizobium japonicum* DSM 30131; *Brevundimonas diminuta* DSM 1635; *Magnetospirillum gryphiswaldense* DSM 6361; *Paracoccus denitri-*

ficans DSM 65; *Rhizobium meliloti* DSM 30135; *Rhodobacter capsulatus* DSM 1710; *Rhodopseudomonas palustris* DSM 123; *Rhodospirillum rubrum* DSM 107.

β-Proteobacteria: *Alcaligenes eutrophus* DSM 531; *Alcaligenes faecalis* ATCC 8750; *Aquaspirillum metamorphum* DSM 1837; *Burkholderia cepacia* DSM 50181; *Chromobacterium violaceum* DSM 30191; *Comamonas testosteroni* DSM 50244; *Sphaerotilus natans* DSM 565; *Thiobacillus acidophilus* DSM 700; *Zoogloea ramigera* WS 1610.

γ-Proteobacteria: *Acinetobacter calcoaceticus* LMG 1046; *Aeromonas hydrophila* WS 1406; *Alteromonas putrefaciens* DSM 50426; *Enterobacter aerogenes* WS 1292; *Enterobacter cloacae* WS 1293; *Erwinia carotovora* WS 1394; *Escherichia coli* DSM 30083; *Leucothrix mucor* DSM 2157; *Proteus vulgaris* WS 1356; *Pseudomonas aeruginosa* DSM 50071; *Pseudomonas alcaligenes* DSM 50342; *Pseudomonas fluorescens* DSM 50090; *Pseudomonas pseudoalcaligenes* LMG 1225; *Pseudomonas putida* DSM 291; *Serratia marcescens* WS 1359; *Vibrio anguillarum* NCIMB 2129.

δ-Proteobacteria: *Myxococcus fulvus* GBF Mx-f2; *Myxococcus virescens* GBF Mx-v4.

Low G+C Gram-positive bacteria: *Bacillus cereus* DSM 31; *Bacillus subtilis* ATCC 6633; *Clostridium acetobutylicum* NCIMB 8052; *Clostridium stercorarium* NCIMB 11754; *Enterococcus faecalis* DSM 20478; *Enterococcus faecium* DSM 20477; *Lactobacillus casei* LMG 9091; *Lactococcus cremoris* DSM 20069; *Lactococcus lactis* DSM 20481; *Pectinatus frisingensis* DSM 20465; *Staphylococcus aureus* DSM 20231; *Staphylococcus carnosus* DSM 20501; *Streptococcus salivarius* DSM 20560.

High G+C Gram-positive bacteria: *Brevibacterium ketoglutamicum* DSM 20165; *Brevibacterium linens* DSM 20425; *Corynebacterium glutamicum* DSM 20300; *Micrococcus luteus* CCM 169; *Nocardioides simplex* DSM 20130; *Propionibacterium freudenreichii* DSM 20271; *Rhodococcus rhodochrous* DSM 43008.

Eukaryotes: *Hansenula anomala* DSM 70255; *Saccharomyces carlsbergensis* WS 66; *Saccharomyces cerevisiae* DSM 70449.

Isolation of total nucleic acids, immobilization on nylon membranes and hybridization with digoxigenin-labelled oligonucleotide probes were done according to Manz *et al.* (1992).

Hybridization stringency of each probe was optimized by gradually increasing the formamide concentration in steps of 5% (v/v) and accordingly lowering the sodium chloride concentration in the washing buffer (Manz *et al.*, 1992). For dot-blot hybridizations with probes CF319a and CF319b, hybridization solution contained 0·9 M NaCl, 20 mM Tris/HCl (pH 7·4), 0·01% SDS, 0·1% *N*-lauroylsarcosine, 4% (v/v) blocking reagent (Boehringer Mannheim), 45% (v/v) formamide and 10 pmol digoxigenin-labelled oligonucleotide. Hybridizations with probe FFE8b were performed with 20% (v/v) formamide. In the washing solutions, hybridization stringency was maintained by lowering the sodium chloride concentration according to the formamide concentration used in the hybridization buffers: for probes CF319a and CF319b the washing buffer contained 20 mM Tris/HCl (pH 7·4), 0·01% SDS, 44 mM NaCl; for probe FFE8b the washing buffer contained 20 mM Tris/HCl (pH 7·4), 0·01% SDS, 250 mM NaCl.

Whole-cell hybridization. Fixed cells immobilized on microscope slides were hybridized by application of 8 µl hybridization solution [0·9 M NaCl, 20 mM Tris/HCl (pH 7·4), 0·01% SDS, 35% (v/v) formamide for probes CF319a and CF319b; 20% (v/v) formamide for probe FFE8b], containing 50 ng probe, to each well of the slide and incubation for at least 1·5 h in an isotonically equilibrated humid chamber at 46 °C. The labelled oligonucleotides were removed gently by rinsing with washing buffer (20 mM Tris/HCl, 0·01% SDS, containing 88 mM NaCl for probes CF319a and CF319b, and 250 mM NaCl for probe FFE8b). Hybridizations using probe BAC303 were performed without addition of formamide in hybridization buffer; the washing buffer contained 20 mM Tris/HCl (pH 7·4), 0·01% SDS and 0·9 M NaCl. Slides were washed at 48 °C for 15 min, rinsed with distilled water, air-dried and mounted in Citifluor (Citifluor, London, UK).

Determination of melting profile of probe CF319a. Fixed cells of *Empedobacter brevis* (LMG 4012) were hybridized with FLUOS-labelled probe CF319a at different hybridization stringencies, adjusted by various concentrations of formamide ranging from 5% to 60% (v/v) in intervals of 5%. For comparison of probe fluorescence intensities, hybridization signals were recorded using a charge-coupled device (CCD) camera (CF 15/2 multi-control, Kappa Messtechnik) attached to a Zeiss Axioplan microscope. The camera was controlled by an IBM-compatible PC equipped with an Optimas image analysis toolbox (BioScan) processing the analogue output signal of the camera via a colour frame grabber (Imaging Technology). To obtain comparable measurements, camera parameters were at the same settings for all recordings. Background correction, threshold setting and processing of the digital pictures were performed according to Trebesius *et al.* (1994). For each formamide concentration, resulting fluorescence intensities of at least 200 cells were analysed and evaluated statistically with Excel 4.0 (Microsoft).

Microscopy and documentation. Probe fluorescence was detected with a Zeiss Axioplan microscope fitted for epifluorescence microscopy with a 50 W high-pressure bulb and Zeiss filter sets 09 and 15. Black-and-white micrographs were taken on Kodak T_{max} 400. Exposure times were 4–15 s for epifluorescence micrographs and 0·01–0·03 s for phase-contrast.

Construction of phylogenetic tree. A phylogenetic tree for the CFB phylum was reconstructed using all currently available sequences of 16S rRNAs and was drawn based on the results of maximum parsimony, maximum likelihood and distance matrix analyses. An initial tree was reconstructed including only sequence data which in comparison with the *E. coli* 16S rRNA do not contain more than 150 ambiguities, applying maximum parsimony and global optimization methods as implemented in the ARB program package. Highly variable alignment positions, different in more than 50% of the selected 16S rRNA sequences, were excluded from the calculations. Partial sequences containing 150–600 ambiguities were added using the maximum-parsimony approach without allowing any change of tree topology.

RESULTS AND DISCUSSION

Design of oligonucleotides

The region from positions 319 to 336 of the 16S rRNA (numbering according to Brosius *et al.*, 1981) was ascertained to be conserved for most of the bacteria belonging to the flavobacteria-cytophaga group. About 80% of the species in this group have a C residue at position 328 of their 16S rRNA sequences; the remainder have a U residue at the corresponding position. Consequently, we synthesized two versions of the oligonucleotide probe CF319: CF319a has a G at position 9 of the probe sequence; CF319b has an A at the corresponding position. The target site of the probe specific for the bacteroides group (BAC303) ranged from positions 303 to 319 and was located immediately upstream of the

Position	299	303	319	340
BAC303		AAGGUCCCCCACAUUG	G	
CF319a			GUACUGAGACACGGACCA	
CF319b			GUACUGAGAUACGGACCA	
Bacteroides vulgatus	GAGG	AAGGUCCCCCACAUUG	G**A**ACUGAGACACGG**U**CCA	AACU
Bacteroides fragilis	GAGG	AAGGUCCCCCACAUUG	G**A**ACUGAGACACGG**U**CCA	AACU
Prevotella oris	GAGG	AAGGUCCCCCACAUUG	G**A**ACUGAGACACGG**U**CCA	AACU
Prevotella nigrescens	GAGG	AAGGUCCCCCACAUUG	G**A**ACUGAGACACGG**U**CCA	AACU
Bacteroides distasonis	GAGG	AAGGUCCCCCACAUUG	GUACUGAGACACGGACCA	AACU
Flavobacterium aquatile	GAGG	**G**AG**A**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Flavobacterium odoratum	GAGG	**G**AG**A**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Porphyromonas circumdentaria	GAGG	**UU**G**AC**CCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Porphyromonas endodontalis	GAGG	**UU**G**AC**CCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Porphyromonas macacae	GAGG	**UUUA**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Cytophaga uliginosa	GAGG	**GG**G**A**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Chryseobacterium gleum	GAGG	**GU**G**A**UCCCCNACA**C**UG	GUACUGAGACACGGACCA	GACU
Empedobacter brevis	GAGG	**GU**G**AA**CCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Flavobacterium meningosepticum	GAGG	**GU**G**A**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Sphingobacterium spiritivorum	GAGG	A**GAA**UCCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Sphingobacterium heparinum	GAGG	A**U**G**AC**CCCCCACA**C**UG	GUACUGAGACACGGACCA	GACU
Saprospira grandis	GAGC	**GU**G**A**UCCCCCACA**CG**G	GUACUGAGACACGGACCN	GACU
Cytophaga hutchinsonii	GAGG	A**U**G**A**UCCCCCACA**C**UG	GUACUGAGAUACGGACCA	GACU
Flavobacterium ferrugineum	GAGC	A**C**G**AC**C**AGU**CACA**CG**G	G**C**ACUGAGACACGG**G**CNN	GACU
Brevundimonas diminuta	GAGG	A**U**G**AC**C**AG**CCACA**C**UG	G**G**ACUGAGACACGG**C**CCA	GACU
Comamonas testosteroni	GAGG	A**C**G**AC**C**AG**CCACA**C**UG	G**G**ACUGAGACACGG**C**CCA	GACU
Escherichia coli	GAGG	A**U**G**AC**C**AG**CCACA**C**UG	G**A**ACUGAGACACGG**U**CCA	GACU
Desulfovibrio desulfuricans	GAGG	A**U**G**A**UC**GG**CCACA**C**UG	G**A**ACUGA**A**ACACGG**U**CCA	GACU
Helicobacter pylori	GAGG	**GU**G**AA**C**GGA**CACA**C**UG	G**A**ACUGAGACACGG**U**CCA	GACU
Bifidobacterium bifidum	GAGG	**GC**G**AC**C**GG**CCACAUUG	G**G**ACUGAGAUACGG**C**CCA	GACU
Bacillus subtilis	GAGG	**GU**G**A**UC**GG**CCACA**C**UG	G**G**ACUGAGACACGG**C**CCA	GACU

Fig. 1. Alignment of target sites of probes BAC303, CF319a and CF319b with the complementary 16S rRNA regions from organisms of the CFB phylum and other main branches of *Bacteria*. Positions that differ from the target sequence are shown underlined in bold type (positions numbered according to Brosius *et al.*, 1981).

Table 2. Oligonucleotide probe sequences, target sites and formamide concentration in the hybridization buffer required for specific whole-cell *in situ* hybridizations

Probe	Sequence	Target site (rRNA positions)*	Formamide (%)
BAC303	5′-CCAATGTGGGGGACCTT-3′	16S (303–319)	0
CF319a	5′-TGGTCCGTGTCTCAGTAC-3′	16S (319–336)	35
CF319b	5′-TGGTCCGTATCTCAGTAC-3′	16S (319–336)	35
FFE8b	5′-CAGCCGCACACCCGTCTT-3′	16S (225–242)	20

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

target for probes CF319a and CF319b (Fig. 1). A region between positions 225 and 242 of the 16S rRNA was found to be characteristic for *Flavobacterium ferrugineum*. Oligonucleotide sequences and target sites are summarized in Table 2. An alignment of target sites of probes BAC303, CF319a and CF319b with the complementary 16S rRNA regions from bacteria representing the CFB phylum and other main branches of *Bacteria* is shown in Fig. 1.

Specificities of oligonucleotide probes

Analysis of the target regions revealed that the sequence of probe CF319a showed 100% similarity to more than 90% of sequences belonging to the flavobacteria-cytophaga group. In addition, species of the genus *Porphyromonas* (Shah & Collins, 1988), namely *P. circumdentaria*, *P. endodontalis*, *P. cansulci*, *P. gingivalis*, *P. salivosa* and *P. macacae* (formerly *Bacteroides macacae*), and *Bacter-*

oides forsythus, which is regarded as being related to the porphyromonas subcluster (Paster *et al.*, 1994), showed 100% sequence similarity to the target region. The same target sequence appeared for the outlying species *Rikenella microfusus* (Collins *et al.*, 1985) and *Bacteroides putredenis*, which can be regarded as an indication of the affiliation of these organisms to the flavobacteria-cytophaga group.

Capnocytophaga ochracea, *Ca. sputigena*, *Blattabacterium* sp., *Flexibacter canadensis*, *Cytophaga hutchinsonii*, *C. aurantiaca*, *Sporocytophaga myxococcoides*, *Microscilla marina*, *Flexibacter elegans*, *F. ruber* and *Spirosoma linguale*, which all have a U residue at position 328 of their 16S rRNAs, showed 100% similarity to the sequence of probe CF319b.

The phylogenetic position of *Bacteroides distasonis* is still under discussion and its affiliation to the genus *Bacteroides* (Shah & Collins, 1989; Shah, 1992) or the porphyromonas cluster (Paster *et al.*, 1994, 1985; Gherna & Woese, 1992) still has to be clarified. Interestingly, *B. distasonis* showed 100% similarity to the target region of probes CF319a and probe BAC303, which reflects the intermediate position of this organism.

Probes CF319a and CF319b showed at least one mismatch to all accessible eubacterial and archaebacterial 16S and 23S rRNA sequences not affiliated to the CFB phylum.

Probe BAC303 showed 100% similarity only to 16S rRNA sequences of the genera *Bacteroides* and *Prevotella*, and at least one mismatch with all other accessible 16S or 23S rRNA sequence data. *Bacteroides splanchnicus*, which has been reported to branch off separately from the other subcluster, but belonging to the bacteroides group (Paster *et al.*, 1994) also showed 100% sequence similarity to the target site of probe BAC303. Comparative analysis of the data set of aligned 16S rRNA sequences revealed only three species of the genus *Prevotella* which did not completely match with the target region of probe BAC303: *Prevotella corporis* has an A residue at position 317; *P. oralis* and *P. loescheii* have a C residue at position 316.

Probe FFE8b showed 100% similarity only to *Flavobacterium ferrugineum*.

Fig. 2 shows a phylogenetic tree reflecting the relationships of members of the CFB phylum and the corresponding sequence similarity of these species to the target sites of probes BAC303, CF319a/b and FFE8b.

In dot-blot analyses, probe CF319a hybridized to all reference strains of the flavobacteria-cytophaga group with the exception of *Bergeyella zoohelcum* LMG 8352, *Cytophaga hutchinsonii* LMG 10844, *Flavobacterium ferrugineum* LMG 4021, *Sporocytophaga myxococcoides* LMG 8393, *Taxeobacter ocellatus* GBF Txo1, and *Weeksella virosa* strains LMG 11602 and LMG 8350 (Table 1). As expected, probe CF319b showed strong hybridization signals to *Cytophaga hutchinsonii* LMG 10844 and *Sporocytophaga myxococcoides* LMG 8393, but there was no signal with any other strain tested. As expected, the species-specific probe FFE8b gave strong hybridization signals only with *Flavobacterium ferrugineum* LMG 4021, and no hybridization signal to any other strains tested (Table 1).

Determination of melting profile of probe CF319a

Using a cooled CCD camera in combination with image analysis software we determined the melting characteristics of probe CF319a to optimize the stringency conditions for application in whole-cell hybridization. The resulting melting profile showed a rapid decrease of fluorescence signals using formamide concentrations higher than 35% (v/v), which confirmed the stringency condition determined empirically in the dot-blot approach. For an optimal signal-to-noise ratio we used 35% (v/v) formamide for *in situ* whole-cell hybridization.

In situ detection of members of the flavobacteria-cytophaga group in activated sludge

Abundant occurrence of members of the flavobacteria-cytophaga group in activated sludge has been detected using conventional techniques. In various water-purification systems, flavobacteria appear to be always present, and in activated sludge plants they were reported as one of the major taxonomic groups, representing up to 60% of the viable flora (Pike, 1975), although many might be more properly regarded as cytophagae (Güde, 1980). However, in view of the prevailing taxonomic uncertainty, it is difficult to make reliable statements about the quantitative occurrence and functional importance of species in these groups for the waste-water treatment process. Analysis of the microbial community structure of activated sludge by *in situ* hybridizations using probe CF319a together with other group-specific oligonucleotides, and by standard cultivation techniques, gave major differences in results, caused by changes in the relative abundance of species upon cultivation (Manz *et al.*, 1994).

Further control hybridizations performed by simultaneous hybridization with differently labelled fluorescent probes for the α-, β- and γ-subclasses of *Proteobacteria* (Manz *et al.*, 1992) and a probe specific for the high-G+C Gram-positive bacteria (Roller *et al.*, 1994) revealed that cells hybridizing with probes CF319a and CF319b did not react with any of the available probes specific for these other phylogenetic groups.

In situ hybridizations of activated sludge samples obtained from different waste-treatment plants showed that members of the flavobacteria-cytophaga group were always present, in amounts ranging from 10% (Hirblingen, FRG), 12% (Berlin-Ruhleben, FRG) and 23% (München Großlappen, FRG) up to more than 50% (Aretsried, FRG) of the cells which could be hybridized with the *Bacteria*-specific probe EUB338.

Interestingly, two different types of cell morphology could be distinguished in activated sludge samples. The first type, which was detected in variable percentages, was characterized by tightly packed, small spindle-shaped cells, typically forming the cores of activated sludge flocs. These new results confirmed earlier findings that flavobacteria may have a direct role as floc-forming organisms (Shewan & McMeekin, 1983) and, due to the known importance of bacterial floc formation, affect the performance of the activated sludge process (Pipes, 1978). In

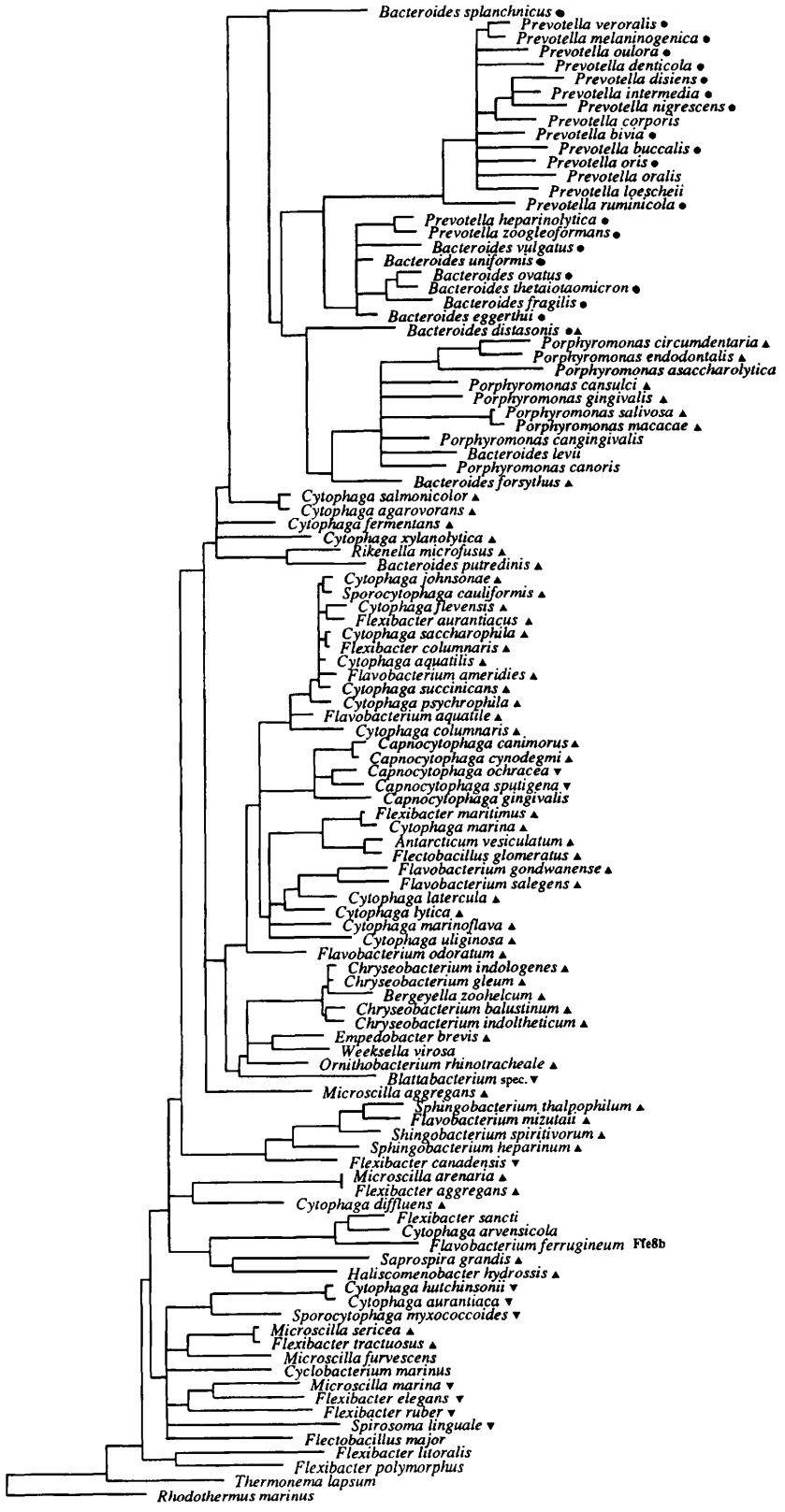

Fig. 2. Phylogenetic tree showing the relationships of members of the CFB phylum and the sequence homologies of the bacteria to the probes BAC303 (●), CF319a (▲), CF319b (▼) and FFE8b (Ffe8b). The branch lengths correlate with the significance of the separation of the respective internal and terminal nodes. Multiple branchings indicate that the relative order of the corresponding branches could not be resolved or was not supported using different methods of tree construction.

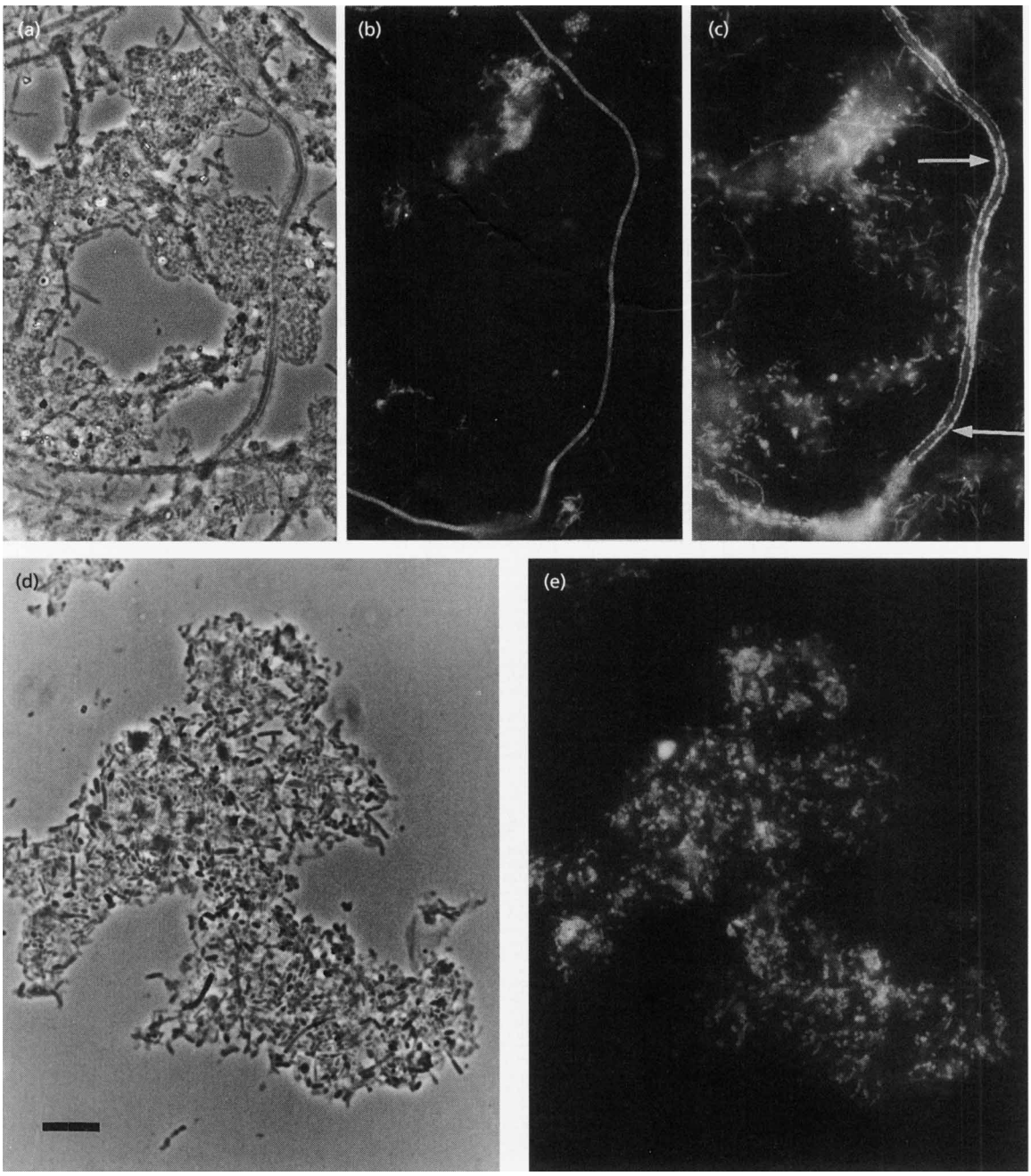

Fig. 3. *In situ* hybridizations of activated sludge from a municipal wastewater treatment plant (a–c) and of human faeces obtained from a healthy adult (d–e). Phase-contrast (a, d) and epifluorescence micrographs (b, c, e) are shown for identical fields. Simultaneous hybridization of an activated sludge floc with a tetramethylrhodamine-labelled probe, specific for the γ-subclass of *Proteobacteria* (b) and the fluorescein-labelled probe CF319a, specific for the flavobacteria-cytophaga group (c), visualized cells colonizing a filamentous bacterium, indicated by arrows. (e) Single bacterial cells within human faeces belonging to the bacteroides group identified by hybridization with the tetramethylrhodamine-labelled probe BAC303. All photomicrographs were taken at a magnification of $\times$ 1000. Bar, 10 μm (d).

contrast to the ability of cytophagae to degrade complex macromolecules, flavobacteria are not known to degrade dextran, lignin or cellulose, but it is probable that they are involved in the breakdown of various proteins and carbohydrates which occur at high concentrations in activated sludge. The second type of morphology exhibited by cells hybridizing with probes CF319a and CF319b is illustrated in Fig. 3(a–c); these cells characteristically colonized the surfaces of inorganic and organic structures. A filamentous bacterium, hybridizing with a probe specific for the γ-subclass of *Proteobacteria* (Fig. 3b), was densely colonized by small rod-shaped cells, which were

arranged in a highly ordered way on the outer sheath of the filamentous organism (Fig. 3c). The strong fluorescence intensity of these colonizing bacteria after hybridization with the fluorescein-labelled probe CF319a (Fig. 3c) is a clear indication of their high metabolic activity. This highly ordered arrangement of two bacterial species could indicate that they form stable consortia based on some type of biochemical or physiological interaction. Further oligonucleotide probes with specificities ranging between group- and species-specificity will be designed for more detailed investigations of this and other structure–function relationships.

In situ hybridization of human faeces

Bacteroides vulgatus, *B. distasonis* and *B. thetaiotaomicron* are the most numerous *Bacteroides* species in the human colon, and normally occur at cell numbers of about 10^{10} per g dry weight of human faeces (Salyers, 1984). These species are all members of the newly reclassified genus *Bacteroides sensu stricto* (Shah, 1992). Cultivation of obligate anaerobes (Moore & Holdemann, 1974) requires stringent anaerobic techniques, and the conventional bacteriological methods for isolating, identifying, and enumerating colonic *Bacteroides* are cumbersome and time-consuming (Moore *et al.*, 1978). To circumvent these limitations, various molecular approaches have been developed for rapid identification of *Bacteroides* strains (Kuritza & Salyers, 1985; Kuritza *et al.*, 1986; Roberts *et al.*, 1987). In contrast to these DNA hybridization techniques, rRNA-targeted oligonucleotides allow not only identification, but also *in situ* analysis and elucidation of the structural arrangement of the target organisms. Fig. 3(e) shows single bacterial cells within human faeces after hybridization with the tetramethylrhodamine-labelled probe BAC303, specific for the genera *Bacteroides* (*sensu stricto*) and *Prevotella*.

Besides the genus *Bacteroides*, Gram-positive bacteria, like *Bifidobacterium* spp., are also present in high numbers in the human colon. By performing a simultaneous *in situ* hybridization of faecal specimens using differently labelled derivatives of the probe HGC, specific for high-G+C Gram-positive bacteria (Roller *et al.*, 1994), and the probe BAC303, we could visualize these two important populations as parts of the normal human faecal flora (data not shown). Further studies are in progress aimed at analysing the spatial arrangement and functional interactions of *Bacteroides* spp. with other bacteria within the complex microbial gut ecosystem.

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