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FEMS Microbiology Ecology 25 (1998) 43–61

FEMS MICROBIOLOGY ECOLOGY

Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides

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Received 9 June 1997; revised 3 October 1997; accepted 6 October 1997

Abstract

A comprehensive panel of ten 16S rRNA targeted oligonucleotides specific for mesophilic sulfate-reducing bacteria (SRB) within the δ-subclass of *Proteobacteria* was developed as a diagnostic tool and evaluated for its specificity and in situ applicability. Five probes (DSD131, DSBO224, DSV407, DSR651, DSS658) are specific on genus level and five probes identify distinct phylogenetic subbranches within the families *Desulfobacteriaceae* (DSMA488, DSB985) and *Desulfovibrionaceae* (DSV214, DSV698, DSV1292). All oligonucleotides were checked for their specificity by computer aided comparative sequence analysis. For in situ application optimal stringency conditions were adjusted for each fluorescence-labelled probe performing whole cell hybridizations using target and non-target organisms. Activated sludge flocs from different stages within the aeration tank of a large municipal wastewater treatment plant were examined by in situ hybridizations with the indocarbocyanine- (Cy3-) labelled SRB-specific probes. The relative abundance and the spatial organization of single SRB were monitored with epifluorescence and confocal laser scanning microscopy. Individual sulfate-reducing cells could be visualized and the number of cells ranged from 0.5 to 8% of the total cell counts within all stages of the activated sludge process and the final clarifier. Cells yielding strong fluorescence signals after hybridization with the newly developed probes were not restricted to anoxic and anaerobic compartments, but were also clearly detectable in the aeration zones of the treatment plant.

Keywords: Sulfate-reducing bacteria; Activated sludge; In situ hybridization

1. Introduction

In the last decade, the phylogeny of sulfate-reducing bacteria (SRB) has been redefined by analyzing 16S ribosomal RNA (rRNA) sequences [1–3]. The Gram-negative mesophilic SRB have been classified into 7 major phylogenetic groups [4]. Further progress in molecular biology did not only provide helpful tools for the investigation of bacterial taxonomy, but was also responsible for recent advances in microbial ecology [5,6]. Detailed analyses of occurrence and abundance of SRB within complex anaerobic microbial communities has formerly been restricted

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0168-6496/98/$19.00
PII S0168-6496(97)00082-2

to information obtained by conventional culture techniques and was biased by the known inability to cultivate most of the contributing organisms [7,8]. Today, the application of direct in situ methods overcomes these limitations and enables microbial communities to be characterized in their natural habitats.

In particular, diagnostic rRNA directed oligonucleotides have been designed for specific groups of SRB [3,9,10] and have been demonstrated to represent valuable tools for group- and species-specific hybridization studies of SRB populations in complex communities such as biofilms [11–14], marine sediments [15] and microbial mats [14,16] without prior isolation of the target organisms.

Parallel to the development of culture-independent methods in microbial ecology, physiological studies revealed the widespread occurrence of SRB. They were not restricted to marine environments, where sulfate reduction predominates over methanogenesis caused by high sulfate concentrations [17–19], but could also be found in freshwater habitats [20,21]. The behaviour of SRB under low oxygen pressure has been studied, and high rates of sulfate reduction within the oxic zones of complex microbial habitats could be demonstrated [22]. In cyanobacterial mats, diurnal cycles of sulfate reduction under oxic conditions could be shown [23]. Additionally, the capacity of several sulfate-reducing species to metabolize different substrates with oxygen as terminal electron acceptor has been studied [24–26].

Since it could be shown for some SRB that they are able to tolerate molecular oxygen [27,28], SRB are increasingly perceived as microaerophiles, rather than obligate anaerobes [29] and, with regard to their ecology, unexpected new activities and abilities could be revealed [22,25,30]. Furthermore, SRB are able to colonize interfaces between oxygen-containing and oxygen-free water layers [31] and the gradient of redox conditions along this interface causes a spatial distribution of the respective microorganisms according to their oxygen tolerance and their mode of metabolism.

Although SRB have been cultivated from well aerated activated sludge [32] and sulfate reduction has been shown in aerobic biofilms [14,33] and in a broad range of aerobic wastewater treatment systems [34], the ecological and technical impact of SRB on the activated sludge process has not yet been investigated in detail.

The objective of this study was the development of a comprehensive panel of oligonucleotides highly specific for mesophilic SRB, that can be used for in situ hybridization. By application of the new, indocarbocyanine- [35] labelled probes, the relative abundance and spatial organization of SRB in activated sludge flocs was studied as an example of technical systems inhabited by complex microbial communities.

2. Materials and methods

2.1. Organisms

The bacterial strains used in this study are given in Table 1.

Pure cultures were obtained from the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), the LMG (Laboratorium voor Microbiologie, Universiteit Gent, Belgium) and GBF (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) and were cultured in media and under culture conditions as recommended in the corresponding catalogues of strains [36,37].

2.2. Sampling

Grab samples (500 ml) of mixed liquor were collected at six sampling sites from the successive anaerobic, anoxic and aeration stages and from the final clarifier at a depth of 1 m within the municipal wastewater treatment plant Berlin-Ruhleben, Germany (1.6 million population equivalents [PE]). For in situ hybridizations, sludge samples were immedi-

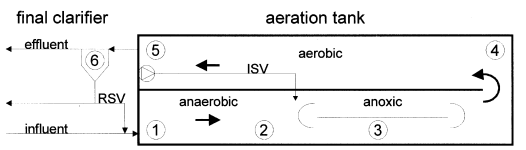

Fig. 1. Schematic process flux within the activated sludge tank and localization of the sampling sites. Abbreviations: ISV, internal sludge flow; RSV, return sludge flow; 1–5, sampling sites.

Table 1
Sources and phylogeny of organisms used in this study

Organism	Source[a]	Phylogeny
Agrobacterium tumefaciens	DSM 30205	α-*Proteobacteria*
Azospirillum brasiliense	DSM 1690	
Brevundimonas diminuta	DSM 1635	
Paracoccus denitrificans	DSM 65	
Rhizobium meliloti	DSM 30135	
Alcaligenes faecalis	DSM 30030	β-*Proteobacteria*
Burkholderia cepacia	DSM 50181	
Comamonas testosteroni	DSM 50244	
Ralstonia eutropha	DSM 531	
Sphaerotilus natans	DSM 565	
Thiobacillus acidophilus	DSM 700	
Acinetobacter calcoaceticus	DSM 30006	γ-*Proteobacteria*
Escherichia coli	DSM 30083	
Legionella pneumophila	DSM 3196	
Pseudomonas aeruginosa	DSM 50071	
Pseudomonas fluorescens	DSM 50090	
Pseudomonas putida	DSM 291	
Myxococcus fulvus	GBFMx-f2	δ-*Proteobacteria*
Desulfobacter sp., strain 3ac10	DSM 2035	
Desulfobacter sp., strain 4ac11	DSM 2057	
Desulfobacter curvatus	DSM 3379	
Desulfobacter hydrogenophilus	DSM 3380	
Desulfobacter latus	DSM 3381	
Desulfobacter postgatei	DSM 2034	
Myxococcus virescens	GBF Mx-v4	
Desulfobacterium autotrophicum	DSM 3382	
Desulfobacterium niacini	DSM 2650	
Desulfobacterium vacuolatum	DSM 3385	
Desulfobacula toluolica	DSM 7467	
Desulfobotulus sapovorans	DSM 2055	
Desulforhopalus vacuolatus	DSM 9700	
Desulfosarcina variabilis	DSM 2060	
Desulfovibrio sp., strain Norway 4	DSM 1741	
Desulfovibrio baarsii	DSM 2075	
Desulfovibrio gigas	DSM 1382	
Desulfovibrio desulfuricans	DSM 642	
Desulfovibrio desulfuricans strain El Agheila Z	DSM 1926	
Desulfovibrio desulfuricans	DSM 6949	
Desulfobulbus sp., strain 3pr10	DSM 2058	
Desulfobulbus propionicus	DSM 2032	
Desulfococcus multivorans	DSM 2059	
Desulfomicrobium baculatus	DSM 1743	
Desulfomonas pigra	DSM 749	
Desulfomonile tiedjei	DSM 6799	
Desulfonema limicola	DSM 2076	
Desulfovibrio longus	DSM 6739	
Desulfovibrio salexigens	DSM 2638	
Desulfovibrio vulgaris ssp. *vulgaris*	DSM 644	
Desulfuromonas acetoxidans	DSM 684	
Pelobacter acetylenicus	DSM 3246	
Pelobacter acidigallici	DSM 2377	
Flavobacterium odoratum	LMG 1233	flavobacteria cytophaga
Cytophaga johnsonae	LMG 1341	

Table 1 (*continued*)

Organism	Source[a]	Phylogeny
Chryseobacterium indologenes	LMG 8337	
Sphingobacterium heparinum	LMG 4024	
Bacillus cereus	DSM 31	Low G+C Gram-positive bacteria
Bacillus subtilis	DSM 10	
Clostridium acetobutylicum	DSM 792	
Clostridium formicoaceticum	DSM 92	
Enterococcus faecalis	DSM 20478	
Enterococcus faecium	DSM 20477	
Corynebacterium glutamicum	DSM 20300	High G+C Gram-positive bacteria
Micrococcus luteus	DSM 20030	
Saccharomyces cerevisiae	DSM 70449	*Eucarya*
Methanosaeta concilii	DSM 2139	*Archaea*
Methanosarcina acetivorans	DSM 2834	
Methanospirillum hungatei	DSM 864	

[a]Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

ately fixed with paraformaldehyde solution (4%) and hybridized according to Manz et al. [38].

The schematic process flux within the aeration tank and the localization of the sampling sites are given in Fig. 1. The average sludge retention time was 3 h in the anaerobic, 6 h in the anoxic and 9 h in the aerobic compartment. Sampling site no. 1 was located at the mixing point near the beginning of the anaerobic zone and site no. 2 at the end of the anaerobic zone. Samples from the anoxic zone were collected at site no. 3, located behind the internal sludge return for nitrate elimination. Sampling site no. 4 was located at the end of the anoxic zone and site no. 5 at the end of the activated sludge process. Sampling site no. 6 was located within the final clarifier. The physico-chemical and operational characteristics of the plant studied are summarized in Table 2.

2.3. Cell fixation

Pure cultures growing in the logarithmic phase were harvested by centrifugation of 2 ml cell suspension (2 min, $5000 \times g$) and resuspended in 1 ml sterile phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4). Cell suspensions of pure cultures and activated sludge samples were fixed for at least 1.5 h at 4°C by addition of 3 volumes of 4% (w/v) freshly prepared paraformaldehyde solution (Sigma, Deisenhofen, Germany) and washed twice with PBS.

Fixed cells were spotted on precleaned microscopic slides (Paul Marienfeld, Bad Mergentheim, Germany), air-dried and subsequently dehydrated with increasing concentrations of ethanol (50%, 80%, and 96%, 3 min each).

Table 2
Physico-chemical and operational characteristics of the wastewater treatment plant Ruhleben (Berlin, Germany)

Parameter[a]	DWF	BOD_5	COD	NH_4-N	SO_4	PT	NO_3-N	NO_2-N	T	SA	MLSS	SVI	ISV	RSV
Unit	[m^3]	[mg/l]	[mg/l]	[mg/l]	[mg/l]	[mg/l]	[mg/l]	[mg/l]	[°C]	[d]	[g/l]	[mg/l]	[–]	[–]
influent	12 000	210	699	38	150.8	8.1	ND[b]	ND	ND	–	–	–	–	–
effluent	–	2	41	0.1	154.2	0.3	0.2	9.5	15	–	–	–	–	–
aeration tank										5	4.2	75	4	2

[a]Abbreviations: DWF, dry weather flow; BOD_5, biochemical oxygen demand; COD, chemical oxygen demand; NH_4-N, ammonia nitrogen; PT, ■; NO_3-N, nitrate nitrogen; NO_2-N, nitrite nitrogen; T, temperature; SA, sludge age; MLSS, mixed liquor suspended solids; SVI, sludge volume index; ISV, internal sludge flow; RSV, return sludge flow.
[b]ND, not determined.

2.4. Design of oligonucleotide probes

Complete 16S rDNA sequences of 67 mesophilic SRB within the δ-subclass of *Proteobacteria* were aligned by computer aided comparison with representatives of other major lineages of *Bacteria* and scanned for putative signature sequences using the Probe_Design tool of the ARB software package, which is available on the World Wide Web at the following URL address: http://www.biol.chemie.tu-muenchen.de/pub/ARB.

The proposed oligonucleotides had to fulfil two requirements: Firstly, the specificity should be as high as possible and no organism outside the intended target group should have complete homology within the target sequence. Secondly, the target position should be located within a region of the 16S rRNA molecule suitable for the in situ use of the derived probe.

Potential candidates for analytical in situ probes were compared to the available dataset of about 6200 complete and partial 16S rRNA sequences obtained from the database of the Technical University Munich, Germany [39] complemented with about 50 16S rRNA sequences of δ-subclass sulfate-reducers obtained from the National Center for Biotechnology Information/GenBank (http://www.ncbi.nlm.nih.gov/ Taxonomy).

Oligonucleotide sequences, target sites, references, formamide concentrations in the hybridization buffer and sodium chloride concentrations in the washing buffer used for in situ hybridizations are given in Table 3.

To test previously published probes specific for sulfate-reducing bacteria, sequences were analyzed for apparent specificity using the Probe_Match tool of the ARB software package.

2.5. Construction of phylogenetic tree

A phylogenetic tree for the sulfate-reducing bacteria investigated was reconstructed using 97 currently available sequences of 16S rRNA. The tree was drawn based on the neighbor joining method of Saitou and Nei [40], as implemented in the ARB program package.

2.6. Whole cell hybridization

Custom synthesized oligodeoxynucleotides were purchased 5′-labelled with fluorescein, emission 518 nm (FLUOS, TIB MOLBIOL, Berlin, Germany) or the indocarbocyanine dye Cy3 [41], emission 570 nm (Biometra, Göttingen, Germany).

Fixed cells immobilized on microscopic slides were hybridized by application of 9 μl hybridization solution (0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% SDS, formamide concentrations as given in Table 3), 1 ng labelled probe was added to each well of the slide and the slides were incubated for at least 1.5 h

Table 3
Oligonucleotide sequences, target sites, references, formamide concentrations in the hybridization buffer and sodium chloride concentrations in the washing buffer required for specific in situ hybridizations with CY3-labelled probes

Probe	Target site (16S rRNA positions)[a]	Probe sequence, 5′→3′	Reference	Formamide concentration (%)	[NaCl] (mM)
DSD131	131–148	CCC GAT CGT CTG GGC AGG	this study	20	250
DSV214	214–230	CAT CCT CGG ACG AAT GC	this study	10	500
221	221–240	TG CGC GGA CTC ATC TTC AAA	[3]	35	88
DSBO224	224–242	GGG ACG CGG ACT CAT CCT C	this study	60	15.6
SRB385	385–402	CGG CGT CGC TGC GTC AGG	[5]	35	88
DSV407	407–424	CCG AAG GCC TTC TTC CCT	this study	50	31.2
DSMA488	488–507	GCC GGT GCT TCC TTT GGC GG	this study	60	15.6
DSR651	651–668	CCC CCT CCA GTA CTC AAG	this study	35	88
DSS658	658–678	TCC ACT TCC CTC TCC CAT	this study	60	15.6
660	660–679	GAA TTC CAC TTT CCC CTC TG	[3]	60	15.6
DSV698	698–717	GTT CCT CCA GAT ATC TAC GG	this study	35	88
DSB985	985–1004	CAC AGG ATG TCA AAC CCA G	this study	20	250
DSV1292	1292–1309	CAA TCC GGA CTG GGA CGC	this study	35	88

[a] *E. coli* numbering [64].

in an isotonically equilibrated humid chamber at 46°C. Unbound oligonucleotides were removed by gently rinsing with washing buffer (20 mM Tris/HCl, 0.01% SDS, NaCl concentration as given in Table 3). Slides were subsequently incubated at 46°C for 15 min with washing buffer, rinsed again with distilled water and mounted using antifading reagent (Citifluor AF2, Citifluor Ltd., London, UK). All hybridization and washing steps were performed at 46°C in the dark.

2.7. Optimization of in situ stringency conditions

Hybridization stringencies of indocarbocyanine-(Cy3-) labelled probes were optimized by in situ hybridizations using target organism(s) and non-target organisms displaying one to three mismatches within the target region. The hybridization stringency was gradually increased by the addition of formamide to the hybridization buffer in concentration steps of 5% (v/v) according to Manz et al. [42].

Hybridization solutions for all Cy3-labelled probes contained: 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% SDS and formamide concentrations ranging from 20–60% as given in Table 3.

The washing buffer for all probes contained 20 mM Tris/HCl (pH 7.4) and 0.01% SDS; the sodium chloride concentration has been adjusted according to the formamide concentration used in the hybridization buffer (Table 3).

2.8. Microscopy and documentation

Epifluorescence microscopy was performed with a Zeiss Axioskop (Oberkochen, Germany) fitted with a 50 W high-pressure bulb and Zeiss light filter set no. 01 for DAPI (excitation 365 nm, dichroic mirror 395 nm, suppression 397 nm), no. 09 for FLUOS (excitation 450–490 nm, dichroic mirror 510 nm, suppression 520 nm) and HQ light filter 41007 (AF Analysentechnik, Tübingen, Germany) for Cy3-labelled probes (excitation 535–550 nm, dichroic mirror 565 nm, suppression 610–675 nm).

Confocal laser scanning microscopy was performed on a TCS4D (Leica, Heidelberg, Germany) equipped with an argon/krypton ion laser (Omnichrome, Chino, USA) to supply excitation for Cy3 (excitation wavelength 568 nm, beam splitter 488/568 nm; detector: long pass filter 530 nm).

Images were obtained with a PL APO 100× oil immersion lens (NA 1.4, Leica, Heidelberg, Germany). The signal-to-noise ratio was improved by employing a line average of 8 to record the XY scans. Image processing and three-dimensional reconstructions were done using the standard software package SCANware version 5.10 (Leica, Heidelberg, Germany) delivered with the instrument. Image data were transferred directly from a high resolution monitor to a 100 ASA negative film (Ilford 100 Delta, Cheshire, UK) using an Imagecorder exposure device (FocusGraphics, Foster City, USA).

For each sampling site and oligonucleotide probe used, at least 10 000 cells obtained from ten independent, randomly chosen microscopic fields were microscopically enumerated at a 1000-fold magnification. The relative abundance of specifically hybridized cells was normalized to the total cell count determined by DAPI staining [38].

3. Results

3.1. Design and evaluation of oligonucleotide probes

Complete 16S rRNA sequences of 67 strains of the main subgroups of mesophilic Gram-negative SRB within the δ-subclass of *Proteobacteria* were aligned with representatives of other major lineages of *Bacteria* and scanned for conserved, diagnostic tracts within these genes. A difference alignment of the oligonucleotides with the complementary 16S rRNA regions of target and non-target species is given in Fig. 2.

Potential signature sequences suitable for in situ probes were compared to the ARB database using the Probe_Match tool of the ARB software package to check for other organisms that may have the identical nucleotide sequences. The computer aided specificity check was supplemented by manual analysis of further 16S rRNA sequences of SRB obtained from the National Center for Biotechnology Information/GenBank. The phylogenetic relationships of the SRB and the sequence homologies of the bacteria to the corresponding oligonucleotide probes are given in Fig. 3.

Probe	Number of mismatches	Organism	16S rRNA target region
DSD131	0	*Desulfovibrio desulfuricans* DSM1926	CCUGCCCAGACGAUCGGG
	2	*Spirochaeta africana* X93928	CCUGCCCAGA**G**GAU**G**GGG
	3	*Desulfovibrio gabonensis* U31080	**U**CUGCCC**U**GA**A**GAUCGGG
DSV214	0	*Desulfomicrobium baculatus* DSM1743	GCAUUCGUCCGAGGAUG
	3	*Desulfobotulus sapovorans* DSM2055	GCAUU**U**GU**UU**GAGGAUG
221	0	*Desulfobacterium autotrophicum* DSM3382	UUUGAAGAUGAGUCCGCGCA
	3	*Desulfobotulus sapovorans* DSM2055	UUUGA**G**GAUGAGUCCGCG**UC**
DSBO224	0	*Desulfobotulus sapovorans* DSM2055	GAGGAUGAGUCCGCGUCCC
	1	*Desulfovibrio desulfuricans* DSM6949	**A**AGGAUGAGUCCGCGUCCC
	2	*Desulfovibrio vulgaris* DSM644	**AU**GGAUGAGUCCGCGUCCC
	3	*Desulfomonas pigra* DSM1926	**AGA**GAUGAGUCCGCGUCCC
DSV407	0	*Desulfovibrio gigas* DSM1382	AGGGAAGAAGGCCUUCGG
	1	*Desulfovibrio halophilus* DSM5663	**C**GGGAAGAAGGCCUUCGG
	2	*Desulfomonas pigra* DSM1926	CGGGA**U**GAAGG**U**CUUCGG
	3	*Desulfovibrio desulfuricans* DSM6949	CGGGA**U**GAAGG**UU**UUCGG
DSMA488	0	*Desulfomonile tiedjei* DSM6799	CCGCCAAAGGAAGCACCGGC
	1	*Desulfovibrio species* M80617	CC**U**CCAAAGGAAGCACCGGC
	2	*Desulfovibrio vulgaris* DSM644	CC**U**CCA**G**AGGAAGCACCGGC
	3	*Desulfosarcina variabilis* DSM2060	CC**A**C**UG**AAGGAAGCACCGGC
DSR651	0	*Desulforhopalus vacuolatus* DSM9700	CUUGAGUACUGGAGGGGG
	1	*Corynebacterium renale* DSM20688	CUUGAGUACUG**N**AGGGG**A**
	2	*Pelobacter acetylenicus* DSM2348	CUUGAGUAC**G**GGAG**A**GGG
	3	*Desulfovibrio* species M80617	CUUGAGU**U**C**G**GGAG**A**GGG
DSS658	0	*Desulfosarcina variabilis* DSM2060	AUGGGAGAGGGAAGUGGA
	1	*Pelobacter acetylenicus* DSM2348	A**C**GGGAGAGGGAAGUGGA
	2	*Pelobacter propionicus* DSM2379	A**C**GGGAGAGGG**U**AGUGGA
	3	*Desulforhopalus vacuolatus* DSM9700	A**CU**GGAG**G**GGGUAGUGGA
660	0	*Desulfobulbus propionicus* DSM2032	CAGAGGGGAAAGUGGAAUUC
	2	*Spirochaeta zuelzerae* DSM1903	CAGAGGGGAAA**C**U**A**GAAUUC
	3	*Desulforhopalus vacuolatus* DSM9700	CAGAGGGGAAAGUGGAAUUC
DSV698	0	*Desulfovibrio desulfuricans* DSM6949	CCGUAGAUAUCUGGAGGAAC
	1	*Desulfovibrio gabonensis* U31080	CCGUAGAUAUC**G**GGAGGAAC
	2	*Desulfobacterium autotrophicum* DSM3382	**U**CGUAGAUAUC**A**GGAGGAAC
	3	*Desulforhopalus vacuolatus* DSM9700	**U**CGUAGAUAUC**G**GGAGGAA**U**
DSB985	0	*Desulfobacter postgatei* DSM2034	CUGGGUUUGACAUCCUGUG
	1	*Spirochaeta stenostrepta* DSM2028	CUGGGUUUGACAUCCUG**A**G
	2	*Desulfovibrio africanus* M37315	CUGGGUUUGACAUCCU**UC**G
	3	*Desulfosarcina variabilis* DSM2060	CUGG**A**UUUGACAUCC**CG**GG
DSV1292	0	*Desulfovibrio vulgaris* DSM644	GCGUCCCAGUCCGGAUUG
	1	*Desulfomonas pigra* DSM1926	G**U**GUCCCAGUCCGGAUUG
	2	*Desulfovibrio acrylicus* U32578	GC**A**UCCCAGUCCGGAU**A**G
	3	*Desulfobacterium autotrophicum* DSM3382	**AUA**UCCCAGUCCGGAUUG

Fig. 2. Difference alignments of the 16S rRNA target regions for the SRB-specific oligonucleotides. Indicated are the sequences for the respective target (0 mismatch) and non-target species (1–3 mismatches). Positions that differ from the homologous sequence are underlined in bold type.

To complement the computer aided probe design, the specificity of each probe was verified under in situ conditions by the reproducible and discriminative staining of target and non-target species. Non-target organisms comprised SRB and cells of other major lineages within the domain *Bacteria* with one, two and three mismatches to the target sequences as well as single representatives of the *Archaea* and *Eucarya*.

For the in situ application of fluorescent dyes with high extinction coefficient and quantum yield such as the indocarbocyanine fluorescent dye Cy3 [41], the hybridization conditions need to be readjusted and should get closer to the dissociation temperature (T_d) of the respective oligonucleotide. In general, in situ hybridizations using Cy3-labelled probes required formamide concentrations at least 20% higher than the same hybridization performed with fluorescein- or tetramethylrhodamine-labelled oligonucleotides. After the optimization of stringency conditions, non-target species displaying one mismatch to the target sequence could be unambiguously separated from the target species. The results of the sequence analysis and whole cell hybridizations are summarized in Table 4. The schematic phylogenetic tree given in Fig. 3 indicates the phylogenetic relationships

Table 4
Target organisms, sources and results of sequence analysis and whole cell hybridization

Organism	Reference	Probe												
		DSV698	DSD131	DSV407	DSV1292	DSV214	DSS658	DSB985	221	DSBO224	DSMA488	660	DSR651
Desulfovibrio sp., strain KRS 1, acc. X93146		+			+								
Desulfovibrio desulfuricans, DSM 6949	#	+			+								
Desulfomonas pigra, DSM 749	#	+											
Desulfovibrio sp., strain KH 2, acc. X93147		+			+								
Desulfovibrio longus, strain16910a, acc. Z24450		+			+								
Desulfovibrio termitidis, DSM 5308		+			+								
Desulfovibrio sp., strain PT-2, acc. M98496		+			+								
Desulfovibrio sp., acc. M80617		+											
Lawsonia intracellularis, acc. U30147		+											
Desulfovibrio sp., acc. U07570		+											
Desulfovibrio sp., acc. U07569		+											
Bilophila wadsworthia, acc. L35148		+			+								
Desulfovibrio sp., strain G3 100, acc. X93994		+											
Desulfovibrio caledoniensis, acc. U53465		+											
Desulfovibrio salexigens, DSM 2638	#	+											
Desulfovibrio bastinii, strain Sebr 4225, acc. U53462		+											
Desulfovibrio sp., strain SEBR 6143, acc. U53463		+											
Desulfovibrio sp., acc. L42995		+			+								
Desulfovibrio halophilus, DSM 5663		+			+								
Desulfovibrio longus, DSM 6739	#	+											
Desulfovibrio longus, acc. X63623		+											
Desulfovibrio gracilis, strain SEBR 6116, acc. U53464		+			+								
Desulfovibrio profundus, DSM 11384		+											
Desulfovibrio desulfuricans, strain El Agheila Z, DSM 1926	#		+										
Desulfovibrio gigas, DSM 1382	#			+									
Desulfovibrio gabonensis, acc. U31080				+									
Desulfovibrio africanus, DSM 2603				+									
Desulfovibrio desulfuricans, DSM 642T	#				+								
Desulfovibrio multispirans, strain L5, acc. M37318					+								
Desulfovibrio vulgaris ssp. *vulgaris*, DSM 644	#				+								
Desulfovibrio sp., strain DMB, acc. X86689					+								
Desulfovibrio desulfuricans, strain C-6, acc. M37314					+								
Desulfovibrio fairfieldensis, acc. U42221					+								
Desulfovibrio sp., strain Norway 4, DSM 1741	#					+							
Desulfomicrobium baculatus, DSM 1743	#					+							
Desulfomicrobium escambiense, strain esc1, acc. U02469						+							
Desulfomicrobium apsheronum, DSM 5918 + B68						+							
Desulfosarcina variabilis, DSM2060	#						+						
Desulfosarcina variabilis, strain 3 be 13, acc. M26632							+						
Desulfobacter sp., strain 3ac10 DSM 2035								+					
Desulfobacter sp., DSM 2057								+					
Desulfobacter hydrogenophilus, DSM 3380	#							+					
Desulfobacter curvatus, DSM 3379								+					
Desulfobacter postgatei, DSM 2034	#							+					
Desulfobacula toluolica, DSM 7467								+					
Desulfobacterium niacini, strain PM4, acc. U51845									+				
Desulfobacterium niacini, DSM 2650									+				
Desulfobacterium vacuolatum, DSM 3385									+				
Desulfobacterium autotrophicum, DSM 3382	#								+				
Desulfobotulus sapovorans, DSM 2055	#									+			
Desulfomonile tiedjei, DSM 6799	#										+		
Desulfovibrio baarsii, DSM 2075	#										+		
Desulfobulbus propionicus, DSM 2032	#											+	
Desulfobulbus sp., DSM 2058												+	
Desulfobulbus elongatus, DSM 2908												+	

Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; acc., GenBank 16S rRNA accession number; +, target organism with complete homology with the probe sequence; #, organisms used for whole cell hybridizations.

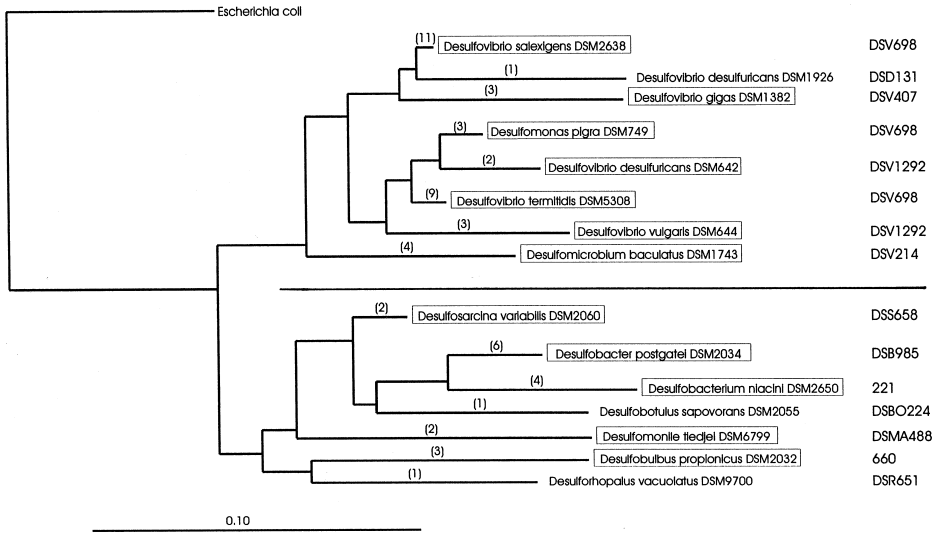

Fig. 3. 16S rRNA based schematic phylogenetic tree reflecting the relationships of SRB within the *Desulfovibrionaceae* (upper branch) and *Desulfobacteriaceae* (lower branch) and the lineages targeted by the respective oligonucleotides. For each lineage one representative strain is indicated, numbers in brackets give the SRB encompassed by the respective probe. The scale bar corresponds to 0.1 substitutions per nucleotide position.

of SRB and the lineages targeted by the respective oligonucleotide probes.

3.2. Oligonucleotides specific for members of the family Desulfovibrionaceae

The largest phylogenetic entity within the δ-*Proteobacteria* sulfate-reducers is formed by various *Desulfovibrio* species, which define for their part separated lineages.

For the family *Desulfovibrionaceae*, the probes DSV698, DSV1292, DSD131, DSV407 and DSV214 were developed and evaluated.

Analysis of the target regions and whole cell hybridizations revealed that probe DSV698 was specific for *Desulfovibrio desulfuricans* (DSM 6949), *Desulfomonas pigra* (DSM 749), *Desulfovibrio salexigens* (DSM 2638), *Desulfovibrio profundus* (acc. U90726), three strains of *Desulfovibrio longus* (DSM 6739, acc. Z24450, acc. X63623), *Desulfovibrio termitidis* (DSM 5308), *Desulfovibrio gracilis* (acc. U53464), *Desulfovibrio bastinii* (acc. U53462), *Desulfovibrio caledoniensis* (acc. U53465), nine strains of the genus *Desulfovibrio* (acc. X93146, acc. X93147, acc. X93994, acc. M98496, acc. M80617, acc. U07570, acc. U07569, acc. U53463, acc. L42995), *Lawsonia intracellularis* (acc. U30147), *Bilophila wadsworthia* (acc. L35148), *Desulfovibrio halophilus* (DSM 5663) and five bacterial species not yet further characterized.

Probe DSV1292 yielded strong hybridization signals after hybridization with the species *Desulfovibrio desulfuricans* (DSM 6949), *Desulfovibrio vulgaris* ssp. *vulgaris* (DSM 644) and *Desulfovibrio desulfuricans* (DSM 642) and showed 100% sequence complementarity to the 16S rRNA target region of *Desulfovibrio halophilus* (DSM 5663), *Desulfovibrio desulfuricans* (acc. M37314), *Desulfovibrio termitidis* (DSM 5308), *Desulfovibrio gracilis* (acc. U53464), *Desulfovibrio longus* (acc. Z24450), *Desulfovibrio multispirans* (acc. M37318), *Bilophila wadsworthia* (acc. L35148), *Desulfovibrio fairfieldensis* (acc. U42221) and five additional *Desulfovibrio* species (acc. X93146, acc. X93147, acc. M98496, acc. X86689 and acc. L42995).

Desulfovibrio desulfuricans strain El Agheila Z (DSM 1926) is neither encompassed by the probe DSV1292 nor by the probe DSV698. Therefore, probe DSD131 was developed which showed 100% homology to the 16S rRNA sequence of this species.

Probe DSV407 was developed for the identifica-

tion of *Desulfovibrio africanus* (acc. 37315) and *Desulfovibrio gigas* (DSM 1382), constituting two distinct branches within the family *Desulfovibrionaceae*. Nevertheless, both have an identical target site within the 16S rRNA between base position 407–424, used for construction of probe DSV407, which additionally comprised the species *Desulfovibrio gabonensis* (acc. U31080).

Probe DSV214 displayed complete homology to the target sequence of *Desulfovibrio desulfuricans* strain Norway 4 (DSM 1741, suggested as *Desulfomicrobium norvegicum*), *Desulfomicrobium apsheronum* (DSM 5918), *Desulfomicrobium baculatus* (DSM 1743) and *Desulfomicrobium escambium* (acc. U02469), which form the genus *Desulfomicrobium* within the family *Desulfovibrionaceae*.

The combined use of probes DSD131, DSV214, DSV407, DSV698 and DSV1292 enabled the in situ characterization of 84% of the family *Desulfovibrionaceae*, for which 16S rRNA data are currently available.

3.3. Oligonucleotides specific for members of the family Desulfobacteriaceae

Five oligonucleotides (DSS658, DSB985, DSBO224, DSMA488 and DSR651) were developed and evaluated specific for the family *Desulfobacteriaceae* (*Desulfosarcina* and relatives, *Desulfobacter* and relatives, *Desulforhopalus vacuolatus*, *Desulfobotulus sapovorans*, *Desulfoarculus-Desulfomonile*).

Probe DSS658 comprised all strains forming the taxon *Desulfosarcina-Desulfococcus*, represented by the species *Desulfococcus multivorans* (DSM 2059) and *Desulfosarcina variabilis* (DSM 2060), which share 92% similarity in their 16S rRNA sequences [43]. One further species, *Desulfonema limicola* (DSM 2076), displayed 88% sequence similarity within the 16S rRNA gene; for this species, sequence comparison revealed one mismatch within the target sequence.

The *Desulfobacter* species comprise a closely related lineage with members sharing more than 95% sequence similarity. Most members of this group could be successfully hybridized with probe DSB985, which displays 100% homology to the target sequence of *Desulfobacter hydrogenophilus* (DSM 3380), *Desulfobacter curvatus* (DSM 3379), *Desulfobacter postgatei* (DSM 2034), *Desulfobacula toluolica* (DSM 7467) and two further members of the genus *Desulfobacter* (DSM 2057, DSM 2035).

For probe 221 [3], specificity analysis revealed four species with complete sequence homology: two strains of *Desulfobacterium niacini* (DSM 2650, acc. U51845), *Desulfobacterium vacuolatum* (DSM 3385) and *Desulfobacterium autotrophicum* (DSM 3382). Together with the oligonucleotide 221, the newly developed probe DSB985 covers the whole *Desulfobacter-Desulfobacterium* lineage.

Desulfobotulus sapovorans has a distinct phylogenetic position within the family *Desulfobacteriaceae* and consequently did not completely match with the target region of probe 221 [3]. Therefore, the oligonucleotide DSBO224 was developed, allowing the specific hybridization of *Desulfobotulus sapovorans* (DSM 2055). For the separate *Desulfoarculus-Desulfomonile* branch within the mesophilic Gram-negative SRB, probe DSMA488 could be designed and hybridized with both species within this lineage, *Desulfoarculus baarsii* (DSM 2075, formerly described as *Desulfovibrio baarsii*) and *Desulfomonile tiedjei* (DSM 6799). Additionally, this probe has 100% homology to the target sequence of two syntrophic δ-subclass *Proteobacteria* (*Syntrophus gentianae*, DSM 8423; *Syntrophus buswellii*, DSM '2612') to which *Desulfoarculus baarsii* and *Desulfomonile tiedjei* are the next relatives [44].

A further subbranch formed by *Desulforhopalus vacuolatus* and three not definitely affiliated strains was comprised by the probe DSR651 hybridizing with all strains within this lineage characterized by the species *Desulforhopalus vacuolatus* (DSM 9700) and two species of the genus *Spirochaeta* (*S. africana* DSM 8902; *S. asiatica* DSM 8901). For two distantly related *Desulfobulbus* species, which share 92% sequence similarity and appear to be phylogenetically more diverse, the formerly published probe 660 [3] could be successfully used for whole cell hybridizations. Sequence comparison revealed for probe 660 complete homology to *Desulfobulbus propionicus* (DSM 2032), *Desulfobulbus elongatus* (DSM 2908) and *Desulfobulbus* sp. (DSM 2058).

In combination with the probes 221 and 660, the five newly developed oligonucleotides comprise 72% of the 16S rRNA sequences currently affiliated to the family *Desulfobacteriaceae*.

3.4. Evaluation of specificity and in situ suitability of formerly published SRB probes

The specificities of formerly published, group-specific oligonucleotides for different lineages of Gram-negative SRB were analysed by computer aided sequence comparison as described for the newly developed probes. Additionally, the oligonucleotides have been labelled with Cy3 and tested for their in situ suitability by whole cell hybridizations. First of all, six oligonucleotides published by Devereux and coworkers [3], namely probes 129, 221, 660, 687, 804 and 814 have been evaluated.

The *Desulfobotulus*-specific probe 660 and a probe specific for the genus *Desulfobacterium* (221), displayed high specificities and could be shown to be well suited for in situ hybridization. They were therefore used for our studies after adjustment of in situ stringency conditions. Probe 687 encompassed different members of the family *Desulfovibrionaceae* and several non-sulfate-reducing species within the δ-subclass of *Proteobacteria* and was therefore not used for further examinations. The fourth probe (129), specific for the genus *Desulfobacter*, showed clear in situ hybridization signals as well. The newly developed probe DSB985, however, additionally comprised the species *Desulfobacter curvatus* (DSM 3379) and *Desulfobacula toluolica* (DSM 7467) and was therefore used for further investigations. Probes 804 and 814 revealed very weak fluorescent in situ hybridization signals even after labelling with Cy3 and could not be successfully used for whole cell hybridizations, which is in accordance with results reported by Ramsing and coworkers [45] and Rabus et al. [10] for the fluorescein derivatives.

The probes SRB385 [9] and SRB385Db [10] comprised a great number of Gram-negative, mesophilic SRB and showed strong in situ hybridization signals. However, sequence comparisons for both probes also revealed a broad range of non-sulfate-reducing bacteria displaying 100% homology within the target sequence [10,45]. Consequently, both oligonucleotides should be applied in combination with more specific probes for subgroups of sulfate-reducing species in a nested hybridization approach [46,47].

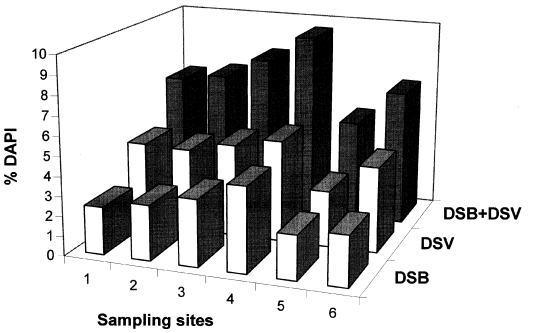

Fig. 4. In situ distribution and abundance of members of the families *Desulfobacteriaceae* and *Desulfovibrionaceae* within the activated sludge process. In situ cell counts obtained with the specific probes were standardized over total cell counts, determined by DAPI-staining. DSB: total of specific counts obtained with probes DSBO224, DSMA488, DSR651, DSS658 and DSB985. DSV: total of specific counts obtained with probes DSD131, DSV214, DSV407, DSV698 and DSV1292.

3.5. Occurrence and quantification of SRB in activated sludge

The relative abundance and spatial distribution of SRB within activated sludge obtained from a municipal sewage treatment plant (Berlin-Ruhleben, Germany) was determined using Cy3-labelled derivatives of the oligonucleotides specific for the family *Desulfobacteriaceae* (probes DSR651, DSB985, DSS658, DSBO224, DSMA488) and the family *Desulfovibrionacae* (probes DSV214, DSV407, DSV698, DSV1292 and DSD131), combined with epifluorescence and confocal laser scanning microscopy.

Individual members of the families *Desulfovibrionaceae* and *Desulfobacteriaceae* could be visualized to different amounts within the activated sludge flocs. The distribution and relative abundance of members of the families *Desulfovibrionaceae* and *Desulfobacteriaceae* determined by in situ probing is shown in Fig. 4.

The number of cells which could be successfully hybridized with the individual *Desulfovibrionaceae*-specific probes ranged from 0.1% (probe DSD131) to 2.4% (probe DSV698) of total cell counts. In the sum, *Desulfovibrionaceae* could be monitored to percentages ranging from 2.8 to 5.2%, depending on the sample sites determined. For *Desulfobacteriaceae*, in situ counts of specific probes ranged from below

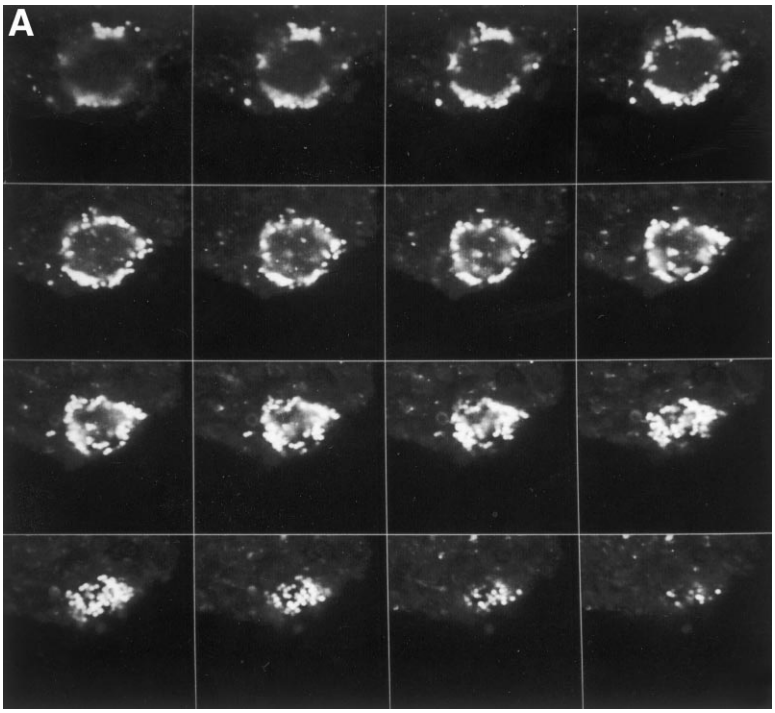

Fig. 5. CLSM micrographs of activated sludge flocs after in situ hybridizations with CY3-labelled SRB probes. Confocal sections were scanned along optical XY axis with increments of 0.5 μm. A: Gallery of 16 optical sections scanned in distances of 0.5 μm representing the spatial distribution of *Desulfobacteriaceae* around the solid core of an activated sludge floc after hybridization with Cy3-labelled probe DSS658. B: Transparent projection of all 24 confocal sections scanned in distances of 0.5 μm resulting in an extended focus image of the microcolony of cells strongly hybridizing with probe DSS658 within the activated sludge floc. C: Transparent projection of confocal sections after hybridization with probes DSV698 and DSV1292 reflecting the disperse arrangement of *Desulfovibrionaceae* within the activated sludge floc. All photomicrographs were done at a magnification of $\times 1000$, bar = 10 μm.

0.1% (probe 660) to 1.8% (probe DSB985) of total cell counts. Summarized, after hybridization of the activated sludge with probes specific for the family *Desulfobacteriaceae*, 5.1 to 9.5% of all DAPI-stained cells showed strong fluorescent signals. During the anaerobic and anoxic treatments of the activated sludge process, in situ counts obtained with the *Desulfobacteriaceae*-specific probes ranged from 2.4 to 4.3%. The number of hybridized cells declined in samples obtained from the end of the aerobic stage to 2.3%; within the final clarifier 2.6% of the total cell counts could be hybridized (sampling site 6). The specific cell counts determined with the *Desulfovibrionaceae*-specific probe were 4.3% (site 1) and 4.2% (site 2) in the anaerobic compartment and 4.7% (site 3) and 5.2% (site 4) in the anoxic zone. As could be shown for members of the family *Desulfobacteriaceae*, the specific cell counts dropped at the end of the aeration zone (2.8%, sampling site 5) and increased again to 4.3% in samples obtained from the final clarifier (sampling site 6).

For probe SRB385, in situ counts ranged from 10.4% (site 1), 8.7% (site 2) and over 10.0% (site 3) to 12.0% at site 4. At the end of the aeration zone (sampling site 5) the SRB385 counts decreased to 7.0% and raised again slightly to 7.9% in activated sludge flocs obtained from the final clarifier.

In view of the spatial distribution, members of the family *Desulfobacteriaceae* were mainly located as part of assemblies or densely packed microcolonies as well as arranged around solid cores within the sludge flocs. Examples typifying the three-dimensional distribution are given in Fig. 5A and B. Using in situ hybridizations with the Cy3-labelled probes DSR651 and DSS658, coccoid bacteria and sarcina-like arranged cells were visualized. These cell

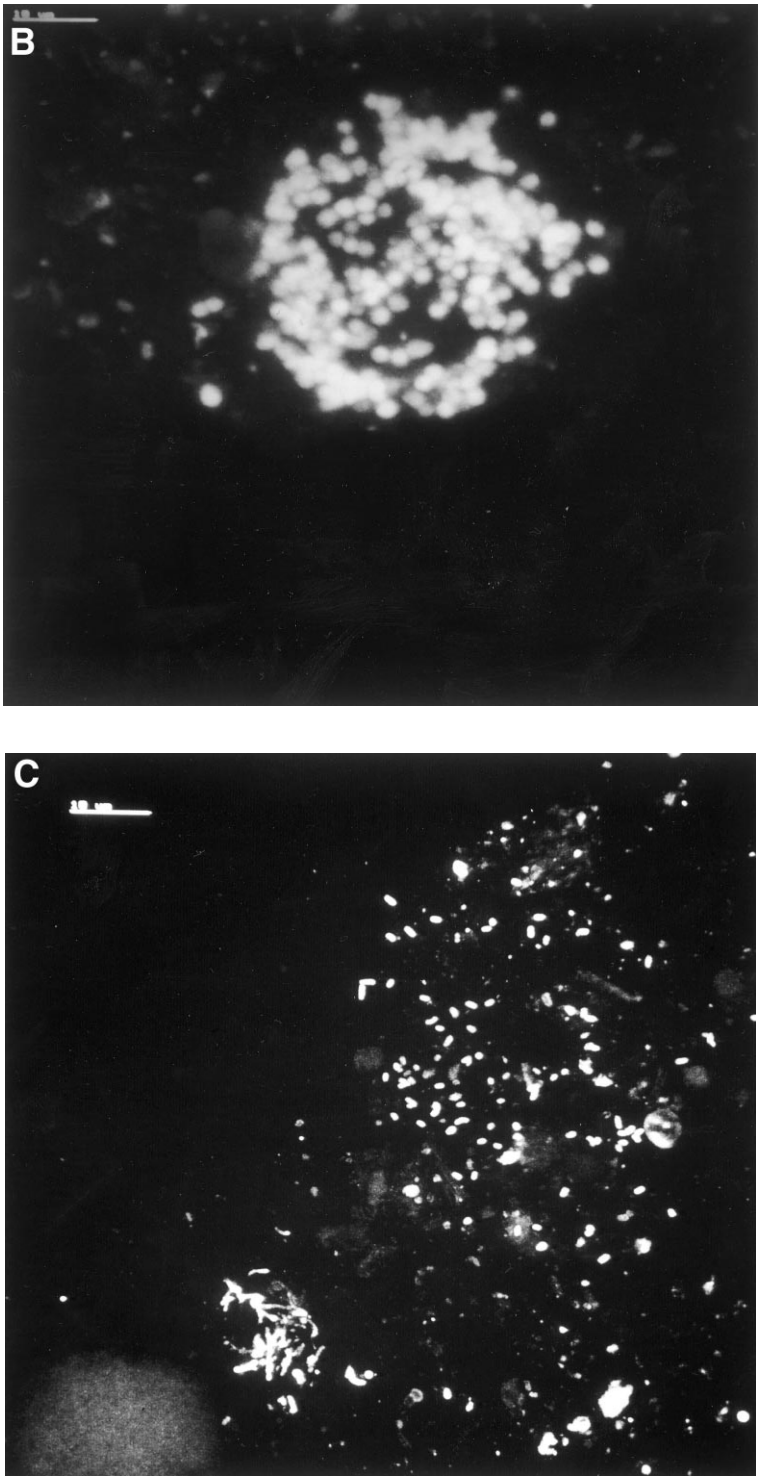

Fig. 5 (*Continued*).

morphologies could not be seen after hybridization with the probe SRB385.

In contrast to the spatial arrangement of the *Desulfobacteriaceae*, individual cells which could be affiliated by in situ hybridization to the family *Desulfovibrionaceae* appeared as slightly curved, vibrio-like or rod-shaped cells. They were mostly arranged as single cells and were homogeneously distributed within the sludge flocs, or to a lesser extent, loosely grouped and attached to inorganic particles. The typical spatial distribution of individual members of the *Desulfovibrionaceae* hybridized with a mixture of Cy3-labelled probes DSV698 and DSV1292 is given in Fig. 5C.

4. Discussion

4.1. Phylogeny of SRB

Comparative 16S rRNA sequence analyses of Gram-negative SRB has placed these organisms into two distantly related phylogenetic lines. One line is composed of thermophilic species, such as *Thermodesulfobacterium*-like organisms, the other lineage contains the mesophilic species. These species belong to the δ-subclass of the *Proteobacteria* [1], comprised of myxobacteria, bdellovibrios and SRB. Recently, this taxon has been expanded by addition of the genera *Pelobacter* [48] and *Geobacter* [49]. Among the Gram-negative mesophilic SRB, two main lineages can be differentiated: the family *Desulfovibrionaceae*, forming a dense phylogenetic cluster of *Desulfovibrio* species ranging at the genus level [2], and the family *Desulfobacteriaceae* [50]. A schematic overview of the phylogenetic relationships among the *Desulfovibrionaceae* and *Desulfobacteriaceae* is given in Fig. 3.

4.2. rRNA approach for in situ analysis of SRB

SRB communities are well suited for rRNA based studies, since the classical physiological based taxonomy is in good agreement with the small subunit rRNA derived phylogeny [2,4]. Therefore the phylogenetic information about SRB within a natural community provided by rRNA techniques allows inferences to be drawn as to the physiological properties and metabolic activities of the corresponding population.

A set of rRNA probes was developed to detect and identify isolated nucleic acids of Gram-negative mesophilic SRB [3,51] performing hybridization assays on membrane blots. However, not all of the oligonucleotides used in these studies are suitable for in situ analyses of microbial communities [45]. For instance, fluorescently labelled probes 804 and 814 [3] revealed very low in situ fluorescence intensities with SRB obtained from culture collections. Since computerized sequence comparisons showed 100% homology of probe 804 to *Mycoplasma* sp. and more than 150 other species which show only one mismatch within the target sequence, this probe has to be used under stringent hybridization conditions, which will further reduce the probe conferred fluorescence signals.

Concerning probe 687 [3], Lonergan [52] recently reported that this probe also hybridized with many Fe(III)-reducing bacteria. In contrast to the above mentioned probes 687, 804 and 814, the group-specific probe SRB385 [9] is principally suited for in situ applications. However, extensive specificity testing revealed that members of the genera *Desulfobacter* and *Desulfomicrobium* as well as all sequenced members of the genera *Desulfobacterium*, *Desulfosarcina*, *Desulfobacula*, *Desulfococcus*, *Desulfoarculus* and *Desulfomonile* displayed at least one mismatch within the target region [45]. Rabus and coworkers [10] therefore suggested a modified version of the probe SRB385, termed SRB385Db, with an altered nucleotide sequence at position 396 (*E. coli* numbering), encompassing all tested members of the family *Desulfobacteriaceae*, except for the species *Desulfobulbus*.

The second severe bias for the application of probes SRB385 and SRB385Db has been revealed by extensive database searches: a large number of bacteria not affiliated to the SRB showed target sequences identical to both probes (e.g. myxobacteria, bdellovibrios, actinomycetes, *Clostridium* species) and more than 1500 species differ only in one mismatch within the target sequence. Many of these are anaerobic bacteria (e.g. *Clostridium* sp., *Pelobacter* sp., *Geobacter* sp.), which inhabit the same ecosystems as sulfate-reducing bacteria.

However, in combination with the comprehensive

panel of specific oligonucleotides developed in this study, probes SRB385 and SRB385b might be successfully applied for in situ hybridizations in a multiple rRNA targeting approach. The combined application of two or more different probes for the identification of one single cell will reduce the probability of identical target sites within variable regions of bacteria not belonging to the intended group of organisms [46] and improves the specificity of in situ probing. Therefore, preliminary results obtained with a probe of broad range specificity, even cross-reacting with a certain number of non-target organisms, can rapidly be validated and confirmed by the subsequent use of specific SRB probes. This nested approach [47], performed with a suite of probes in combination with the two formerly published oligonucleotides 221 and 660 [3] allows the reliable in situ identification of Gram-negative, sulfate-reducing bacteria in natural and technical microenvironments.

Due to the fact that environmental conditions influence the cellular rRNA content [53,54], the amount of rRNA is considered to correlate with the growth rate [13,55], and in situ probing using rRNA targeted oligonucleotides might therefore be a powerful tool for the assessment of bacterial activities in their natural habitats. This is an important advantage over other indirect molecular tools, such as PCR and immunofluorescence, where a distinction between metabolically active and largely inactive cells is not possible.

4.3. Use of high performance fluorochrome Cy3

The use of fluorescent dyes with high quantum yields and extinction coefficients, e.g. Cy3 [41], significantly improves the signal-to-noise ratio of fluorescent assays. Thereby it is possible to overcome the most frequently mentioned limitation of in situ probing, namely too low fluorescence intensity per mole fluorochrome for the detection of target cells with low cellular rRNA contents. High background fluorescence caused by inorganic particles and detritus, another often discussed limitation, can be reduced by a combination of in situ probing with confocal laser scanning microscopy and image processing. On the other hand, the use of high performance fluorochromes raises the background signal of cross-reacting specimens, too. This is caused by the detection of low amounts of fluorochrome-coupled oligonucleotides forming incompletely matched oligonucleotide-rRNA hybrids. To prevent false-positive counts of target cells in natural environments, the renewed assessment of hybridization conditions and the adjustment of in situ stringency using oligonucleotides coupled with these kinds of fluorescent dyes is needed.

4.4. Specificity check

Presently all 16S and 23S rRNA targeted oligonucleotides are constructed based on sequences of culturable organisms and the successful application of the probes depends on the existence of a corresponding reference collection. The isolation of further representatives of the investigated taxon, e.g. SRB, is therefore necessary for the continual evaluation and up-dating of formerly developed rRNA hybridization probes. Information about a certain microbial population, obtained by in situ probing with specific oligonucleotides coupled to high performance fluorescent dyes, could help to monitor and increase the cultivation efficacy of these bacteria from natural ecosystems.

In this study, two strategies have been used for evaluation of probe specificity. Firstly, the apparent specificity of each oligonucleotide was determined by matching it with a comprehensive database of 16S rRNA sequences. The Probe_Match function of the ARB software package generated a list of sequences containing subsequences with up to three base mismatches compared to the target sequence, and indicated the sites of sequence mismatch. With this computational specificity analysis, sequences from a wide variety of organisms can be compared, and the effects of multiple cumulative or independent changes in individual sequences can be tested. Secondly, the in situ specificity of each Cy3-labelled oligonucleotide was adjusted by extensive whole cell hybridizations using target organisms and bacteria with one to three mismatches within the target sequence.

4.5. In situ identification of SRB in the activated sludge process

The occurrence of SRB in wastewater treatment plants has been known for some years [32]. Recent

studies performing in situ analysis of the microbial population involved in the activated sludge process, however, have been mainly focused on aerobic or facultatively anaerobic bacteria [38,56].

In this investigation, in situ probing was performed to determine the occurrence and relative abundance of SRB at different stages of the activated sludge process. By this approach, individual members of the *Desulfobacteriaceae* and *Desulfovibrionaceae* could be shown to be present within the anaerobic, anoxic as well as aerobic zones of the treatment process.

A comparison between the percentage of bacteria yielding hybridization signals with the general probe SRB385 and the amount of bacteria hybridizing with the probes specific for the *Desulfobacteriaceae* and *Desulfovibrionaceae* revealed an equal amount of hybridizing cells. However, since the sequence of probe SRB385 is not complementary to most *Desulfobacteriaceae* [10], the in situ counts obtained with probe SRB385 are expected to severely underestimate the actual amount of SRB within the microbial community. The high number of bacteria hybridizing with the probe SRB385 must therefore be assumed to be due to false-positive bacteria not belonging to the SRB.

The fairly high percentage (6.7%) of SRB-specific counts at the beginning of the activated sludge process shown by in situ probing reflects the viability of SRB even under not strictly anaerobic conditions. During the anaerobic stage, with an average sludge retention time of 3 h, the amount of cells hybridizing with the probes specific for the family *Desulfovibrionaceae* remained constant at percentages of about 4% of the total cell counts. Within the anoxic compartment the relative abundance increased to 5.2%. Because of the aerobic conditions dominating for 9 h in the aerobic compartment of the treatment plant, the relative abundance of *Desulfovibrionaceae* dropped to 2.8% of the total cell counts. However, after the *Desulfovibrionaceae* returned again to the oxygen depleted environment in the final clarifier and stayed in this compartment for about 12 h, the specific in situ cell counts rose to 4.3% of the total bacterial population. This increase in specific counts may be either due to the activation of resting SRB and their subsequent growth or by the inactivation and decease of other bacteria within the activated sludge.

Members of the family *Desulfobacteriaceae* could be hybridized within the anaerobic and anoxic zones to increasing relative amounts ranging from 2.4% at the beginning of the anaerobic zone to 4.3% at the end of the anoxic compartment. Besides the above mentioned decrease of bacteria not affiliated to the SRB, even in this case the reactivation of the metabolic activity could be a possible explanation for the increase in the relative abundance.

Parallel to the drop of cells hybridizing with the *Desulfovibrionaceae*-specific probes, in situ counts for *Desulfobacteriaceae* decreased during the aerobic period of the treatment process. In comparison with the *Desulfovibrionaceae*, however, members of the *Desulfobacteriaceae* seemed to require a prolonged lag phase for the reactivation of their metabolic activity, correlated to the only slight increase of specific counts within the final clarifier to 2.6% of total cell counts.

The various responses of *Desulfobacteriaceae* and *Desulfovibrionaceae* to oxygen stress could also be one possible explanation for the different patterns of the three-dimensional arrangement of both groups. Individual cells strongly hybridizing with the *Desulfovibrionaceae*-specific probes were homogeneously distributed within the activated sludge flocs, whereas members of the *Desulfobacteriaceae* were typically arranged in microcolonies, which putatively allowed them to form oxygen depleted microniches. These observations are in good accordance with results reported by Fukui and Takii [28], which showed that survival of particle-associated SRB was higher compared to free-living cells under aerobic conditions.

Sulfate reduction and sulfide removal activities are ubiquitous in aerobic wastewater treatment systems and the occurrence of SRB throughout the activated sludge process suggests an important ecological function of SRB in aerobic wastewater treatment. For the anaerobic zones of enhanced biological phosphate removal systems, decrease of sulfate concentrations has been reported [57,58] and in situ re-oxidation of H_2S establishes a sulfur cycle which can contribute to the organic matter removal of wastewater treatment plants [34,59]. Kühl and Jørgensen [33] reported a potential contribution of SRB to the organic matter removal in a trickling filter of up to 50%. However, a much lower potential contribution

(less than 2%) was estimated for the conventionally aerated activated sludge.

Microbial communities inhabiting wastewater treatment systems contain both aerobic and anaerobic populations. This versatility can be used for practical applications. The presence of aerobic populations in anaerobic sludges allowed granulation mediated by the interactions between SRB and *Beggiatoa* sp., shown in a multi-stage reverse flow reactor treating domestic sewage [60]. For aerobic wastewater treatment systems, the possible influence of toxic amounts of H_2S on the growth of filamentous microorganisms has been discussed [61].

In the future, a combination of in situ probing, confocal laser scanning microscopy and characterization of physiological parameters by use of selective inhibitors [62], radioactively labelled compounds [63] and microelectrodes [33] will help to assess the abundance, spatial distribution, metabolic potential and ecological function of SRB within the activated sludge process directly at their site of action without prior cultivation. It will be of special interest to find out if the formation of H_2S influences the growth of filamentous bacteria in the sludge flocs and which implication this growth will have on the structure and subsequently on the settling behaviour of the activated sludge flocs.

Acknowledgments

This work was supported by a grant of the Technical University Berlin to W.M. (FIP 6/41). We thank the Berliner Wasserbetriebe for providing the operational characteristics of the treatment plant Berlin-Ruhleben.

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