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Detection of sphingomonads and *in situ* identification in activated sludge using 16S rRNA-targeted oligonucleotide probes

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The increasing significance of members of the genus *Sphingomonas* in biotechnological applications has led to an increased interest in the diversity, abundance and ecophysiological potential of this group of Gram-negative bacteria. This general focus provides a challenge to improve means for identification of sphingomonads; eg molecular genetic methods for rapid and specific detection could facilitate screening of new isolates. Here, fluorescently labeled oligonucleotide probes targeted against 16S rRNA were used to typify strains previously assigned to the genus. All 46 sphingomonads tested including type strains of 21 *Sphingomonas* species could be detected with a probe originally designed for the genus and all but one with a probe designed for the alpha-4 subgroup of the Proteobacteria. The two probes are suitable for direct detection of sphingomonads in pure and mixed cultures as well as in environmental samples of unknown composition. The probes were used to identify sphingomonads *in situ* in activated sludge samples. Sphingomonads were rather abundant accounting for about 5–10% of the total cells in municipal sludges. Distinct patterns in aggregation of the cells suggest that these organisms could be involved in the formation process of sludge flocs.

Keywords: fluorescent *in situ* hybridization (FISH); gene probes; activated sludge; floc structure; alpha-4 Proteobacteria

Introduction

In recent years studies on isolates obtained from environmental specimens resulted in a significant increase in knowledge about the taxonomy of the genus *Sphingomonas*. Sphingomonads are biotechnologically interesting organisms eg because of degradation capabilities for various xenobiotic substances [6,9,13,14,22,37]. Furthermore, some strains have been reported which have the potential to produce exopolysaccharides [5,17,23,24,40]. A significant number of strains has been isolated from materials of clinical origin [38].

The application of specialized *Sphingomonas* strains eg in sphingan production or *in situ* remediation of contaminated aquifers or soils asks for specific and reliable means to rapidly detect and quantify cell populations. Furthermore, such detection methods could be helpful for screening collections of environmental isolates for the presence of sphingomonads.

One widely applied method for rapid identification of bacteria is nucleic acid hybridization using rRNA-targeted oligonucleotide probes [1,28]. Certain sequence signatures within the rRNA molecules are used as specific target sites for probes on eg group or genus level [16]. With the fluorescence-*in-situ*-hybridization (FISH) technique, single cells can be directly identified in laboratory or environmental samples [2,4]. This cultivation-independent method permits determination of natural abundances of community members. Different aquatic and terrestrial samples have been analyzed [10,18,21,41] with a special interest in wastewaters due to their technical significance and high bacterial diversity [20,27,32,33]. The FISH technique suits well for analysis of activated sludge because the large majority of all cells are readily detectable with universal probes [2,33]. Analysis of composition of communities could lead to new insights into the involvement of distinct organisms in processes of nutrient removal from wastewaters.

Phylogenetically the genus *Sphingomonas* belongs to the alpha-4 subgroup of the Proteobacteria wherein it is the dominant cluster apart from a smaller one constituted by the genera *Erythrobacter*, *Erythromicrobium*, and *Porphyrobacter* [31,38]. Other bacteria such as strains of *Blastomonas*, *Rhizomonas*, and *Zymomonas* are intermixed among the *Sphingomonas* cluster. Therefore, they could not be easily distinguished from *Sphingomonas* spp based on 16S rRNA sequence data. Regarding the considerable increase in new species of *Sphingomonas*, nomenclature of members of the cluster is changing rapidly. Recently, some of these organisms eg *Blastomonas natatoria* were proposed to be transferred into the genus *Sphingomonas* [39]. 16S rRNA-targeted probes to detect sphingomonads have been developed which are specific for the alpha-4 subgroup (including the *Erythrobacter-Porphyrobacter* cluster) and for the genus *Sphingomonas* [19]. The latter probe also detects other members of the *Sphingomonas* cluster, eg *Zymomonas*. However, the specificity of these probes has so far been tested only with a small number of strains.

In this study, we confirm the diagnostic value of the previously described oligonucleotides by probing a larger collection of strains representing the *Sphingomonas* cluster. In

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a second step, the probes were used to analyze the presence of sphingomonads *in situ* in activated sludges.

Material and methods

Media and cultivation

Organisms used in this study and the conditions for their cultivation are shown in Table 1. *Sphingomonas* strains were grown either in Nutrient broth (Liebig's meat extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1}, sodium chloride 5 g L^{-1}, pH 7.4) or in R2A broth [25] (yeast extract 0.5 g L^{-1}, protease peptone No. 3 (Difco, Detroit, MI, USA) 0.5 g L^{-1}, casein hydrolysate (Fluka, Sigma Chemical Co, St Louis, MO, USA) 0.5 g L^{-1}, glucose 0.5 g L^{-1}, soluble starch 0.5 g L^{-1}, pyruvate 0.3 g L^{-1}, K_2HPO_4 0.3 g L^{-1}, $MgSO_4 \cdot 7\ H_2O$ 0.05 g L^{-1}, pH 7.2 by KH_2PO_4/K_2HPO_4) depending on their requirement for nutrients. The two *Zymomonas* strains were cultivated in DSMZ medium No. 10 (proteose peptone No. 3, 10 g L^{-1}, yeast extract 10 g L^{-1}, glucose 20 g L^{-1}, pH 7.0) and *Erythrobacter longus* in Marine broth 2216 (Difco). Not all *Sphingomonas* strains could be cultivated in nutrient broth. However, all were able to grow in R2A broth. Cultures were incubated aerobically on a shaker at temperatures of 25, 28, or 30°C due to different sensitivities against temperatures higher than 25°C.

Cell harvest and fixation

Harvest of cells was done from batch cultures in the exponential phase of growth (OD_{600} 0.3–1.0) to ensure a high content of ribosomes per cell. Harvesting was done by centrifugation of 2-ml aliquots for 4 min at 12 000 × *g*. Cell pellets were washed with 1 ml PBS buffer (130 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.2), again centrifuged for 4 min and subsequently resuspended in 200 μl PBS. Fixation was performed by addition of three volumes of a 4% paraformaldehyde (PFA) solution (w/v in PBS, pH 7.2) [3]. The fixates were incubated between 30 min and 16 h at 4°C. Samples were centrifuged for 5 min at 12 000 × *g*, the fixative discarded and pellets again washed with 1 ml PBS. Finally, the fixed cells were thoroughly resuspended in 200 μl PBS, mixed with the same volume of ethanol (99.8%, p.a.) and stored at −20°C.

Fixation of activated sludge samples

Mixed liquor samples of 50 ml activated sludge were taken directly from aeration basins of six wastewater treatment plants (WWTPs) located in central Hesse in the surroundings of Giessen, Germany: Butzbach [municipal, one aerobic stage, 50 000 population equivalents (PE)]; Dorf-Güll (municipal, aerobic pond, 3500 PE); Lich (industrial, brewery sewages, upflow anaerobic sludge reactor and subsequent aerobic stage, 130 000 PE); Oppershofen (municipal, one aerobic stage, 2000 PE); Steinbach (municipal, one aerobic stage, 5000 PE); Wetzlar (municipal, one aerobic stage, 80 000 PE). One PE is defined as a pollution level of 60 g BOD day^{-1} (BOD: 5-day biological oxygen demand) in the incoming wastewater [11].

Aliquots of the original samples were immediately fixed with three volumes of ice-cold 4% PFA solution or an equivalent volume of ice-cold ethanol (99.8% p.a.). Fixed and original samples were transported to the laboratory on ice. Final fixation of PFA-prefixed sludge samples was performed as described above for cultivated cells. Fixed material was stored at −20°C.

Hybridization with fluorescently-labeled oligonucleotide probes and DAPI-staining

Whole cell hybridizations of fixed cultivated cells and *in situ* hybridizations of activated sludge samples were done with the fluorescently-labeled probes EUB338 [3], ALF968, ALF4-1322 and SPH120 [19]. Fluoresceine and carboxy-tetramethylrhodamine-labeled oligonucleotides were purchased from Interactiva (Ulm, Germany). Probes were stored in 10 mM Tris/HCl, 1 mM EDTA buffer, pH 7.2 in concentrations of 50 ng μL^{-1}.

Fixed material (3 μl of cell suspension and 3–10 μl of fixed activated sludge material) was spotted on epoxide-coated six-field slides (Paul Marienfeld GmbH, Bad Mergentheim, Germany). Following a drying step (5 min, 46°C), slides were dehydrated in a series of ethanol baths in an ascending order of concentration (50%, 80% and 96%) for 3 min each. Hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris/HCl pH 7.4, 0.01% sodiumdodecylsulfate (SDS) and the amount of formamide necessary for each probe to perform stringent hybridizations. Stringent conditions ensure that probes bind specifically only to target cells. The amounts of formamide applied were 20% (v/v) for EUB338 and ALF968 and 35% (v/v) for ALF4-1322 and SPH120. Ten microlitres of hybridization buffer containing 50 ng of each probe were dropped on single wells of the slide and dissipated carefully. The slides were positioned horizontally in 50-ml falcon tubes equilibrated isotonically with hybridization buffer and then incubated for 1.5 h at 46°C. Post-hybridization washing was performed at slightly elevated stringency (48°C). Washing buffer consisted of 20 mM Tris/HCl pH 7.4, 5 mM EDTA, 0.01% SDS and a reduced amount of NaCl, equaling the respective amount of formamide [35], 225 mM in the case of 20% formamide and 80 mM for 35%. Slides were quickly rinsed with 1 ml pre-warmed buffer followed by an immersion for 20 min. After rinsing them with distilled water, slides were examined or stained with DAPI for visualization of total cells.

FISH of activated sludges with EUB338 was done with ethanol-fixed material enabling detection of a higher proportion of bacterial cells [26,33]. All other probes were applied to PFA-fixed material.

Counter-staining with the DNA-intercalating dye 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim, Mannheim, Germany) was performed as described previously [21]. Single wells were covered with 1 μg μL^{-1} DAPI in PBS for 10 min in the dark and subsequently rinsed with distilled water. After air-drying, the slides were immediately analyzed microscopically or, alternatively, stored at −20°C for later examination.

Epifluorescence microscopy

Slides were embedded with Citifluor (Citifluor Ltd, Kent, UK) to slow down bleaching of fluorescent dyes and examined at a Zeiss Axioplan 2 microscope (Carl Zeiss GmbH,

Table 1 Organisms, cultivation conditions and typifying results by whole cell hybridization

Organism	Strain designation, obtained from	Medium, temperature (°C)	Fluorescence signal with probe		
			ALF968	ALF4-1322	SPH120
Sphingomonas paucimobilis[1]	NCTC 11030^{T}, (a)	Nutrient, 30	+	+	+
S. paucimobilis	LMG 4017, (b)	Nutrient, 30	+	+	+
S. paucimobilis	EPA505, (c)	Nutrient, 30	+	+	+
S. paucimobilis	BA2, (d)	R2A, 25	+	+	+
S. adhaesiva[1]	IFO 15099^{T}, (d)	Nutrient, 30	+	+	+
S. aromaticivorans	SMCC F199^{T}, (d)	Nutrient, 30	+	+	+
S. asaccharolytica	DSM 10564^{T}, (d)	R2A, 30	+	−	+
S. capsulata[1]	DSM 30196^{T}, (d)	Nutrient, 30	+	+	+
S. chlorophenolica	ATCC 33790^{T}, (e)	Nutrient, 30	+	+	+
S. echinoides	DSM 1805^{T}, (d)	R2A, 30	+	+	+
S. herbicidovorans	DSM 11019^{T}, (d)	Nutrient, 30	+	+	+
S. macrogoltabidus	IFO 15033^{T}, (d)	Nutrient, 30	+	+	+
S. mali	IFO 15500^{T}, (d)	R2A, 30	+	+	+
S. natatoria[1]	DSM 3183^{T}, (d)	Nutrient, 30	+	+	+
S. parapaucimobilis[1]	DSM 7463^{T}, (d)	Nutrient, 30	+	+	+
S. pruni	IFO 15498^{T}, (d)	R2A, 30	+	+	+
S. rosa	IFO 15208^{T}, (d)	R2A, 30	+	+	+
S. sanguinis	IFO 13937^{T}, (d)	Nutrient, 30	+	+	+
S. subarctica	KF1^{T}, (d)	Nutrient, 30	+	+	+
S. subterranea	SMCC B0478^{T}, (d)	Nutrient, 30	+	+	+
S. terrae	IFO 15098^{T}, (d)	Nutrient, 30	+	+	+
S. trueperi	ATCC 12417^{T}, (d)	Nutrient, 30	+	+	+
S. xenophaga	BN6^{T}, (d)	Nutrient, 30	+	+	+
S. yanoikuyae[1]	IFO 15102^{T}, (d)	Nutrient, 30	+	+	+
S. yanoikuyae	DSM 6900, (c)	R2A, 25	+	+	+
S. yanoikuyae	DSM 7235, (d)	Nutrient, 30	+	+	+
S. yanoikuyae	SS3, (f)	Nutrient, 25	+	+	+
Sphingomonas sp	A175, (f)	Nutrient, 30	+	+	+
Sphingomonas sp	A28-24-1, (e)	Nutrient, 30	+	+	+
Sphingomonas sp	C28-24-1, (e)	R2A, 30	+	+	+
Sphingomonas sp	C28-24-2, (e)	R2A, 30	+	+	+
Sphingomonas sp	DSM 7135 (=HH69), (f)	R2A, 30	+	+	+
Sphingomonas sp	DSM 6014 (=RW1), (f)	Nutrient, 30	+	+	+
Sphingomonas sp	E3, (g)	R2A, 25	+	+	+
Sphingomonas sp	EDIV, (d)	Nutrient, 30	+	+	+
Sphingomonas sp	K101, (d)	Nutrient, 25	+	+	+
Sphingomonas sp	MA101b, (e)	R2A, 25	+	+	+
Sphingomonas sp	MA306a, (e)	R2A, 25	+	+	+
Sphingomonas sp	MA405, (e)	R2A, 25	+	+	+
Sphingomonas sp	MAolki, (e)	R2A, 25	+	+	+
Sphingomonas sp	olki/96, (d)	R2A, 25	+	+	+
Sphingomonas sp	NW12, (d)	R2A, 30	+	+	+
Sphingomonas sp	RW5, (f)	Nutrient, 30	+	+	+
Sphingomonas sp	Y9, (g)	R2A, 25	+	+	+
Zymomonas mobilis subsp *mobilis*[1]	DSM 424^{T}, (h)	DSM10, 30	+	+	+
Z. mobilis subsp *pomaceae*[1]	DSM 448^{T}, (h)	DSM10, 30	+	+	+
Erythrobacter longus[1]	ATCC 33941^{T}, (i)	Marine medium, 28	+	+	−
Brevundimonas diminuta[1]	DSM 1635^{T}, (h)	Nutrient, 30	+	−	−

[1]Corresponding results have also been reported in reference [19].
Abbreviations: ^{T}, Type strain; ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFO, Institut for Fermentation, Osaka, Japan; LMG, Laboratorium voor Mikrobiologie, University of Gent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Library, London, UK; SMCC, Subsurface Microbial Culture Collection, Florida State University, Tallahassee, Florida, USA. The strains were derived from the following sources: (a) NCTC; (b) LMG; (c) B Mahro, Fachbereich Bauingenieurwesen, Hochschule Bremen, Germany; (d) H-J Busse, Institute for Bacteriology, Mycology, and Hygiene, Veterinary Medical University of Vienna, Austria; (e) M Salkinoja-Salonen, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland; (f) R-M Wittich, Gessellschaft für Biotechnologische Forschung (GBF), Department of Microbiology, Braunschweig, Germany; (g) Institut für Angewandte Mikrobiologie, University of Giessen, Germany; (h) DSM; (i) ATCC.

Göttingen, Germany) equipped with a 100-W mercury bulb. Emission of the distinct fluorescent dyes was observed with the following filtersets: fluoresceine, 09 (Zeiss) and Chroma HQ-FITC (AHF Analysentechnik, Tübingen, Germany), rhodamine, 15 (Zeiss) and Chroma HQ-Cy3 (AHF Analysentechnik), and DAPI, 01 (Zeiss). Photo-documentation was done with Kodak TMAX 400 black-and-white film. Exposure times were 0.05 s for phase contrast and 4–8 s for epifluorescence pictures. Relative abundances of cells stained with a distinct probe were obtained by comparative cell counts of probe-positive *vs* DAPI-stained cells in identical sections of sample material. Counting was per-

formed using a 10×10-fields grid ocular (Zeiss). For each datum (fraction of probe-positive cells) a constant number of grid fields, depending on the density of the sludge flocs, for 10–20 randomly chosen microscopic fields were examined. For each probe between 2500 and 5500 DAPI-stained cells were counted.

Results

Typing of strains with alpha-proteobacterial probes

Three probes with nested specificity for the *Sphingomonas* cluster, SPH120, the alpha-4 subgroup of the Proteobacteria, ALF4-1322, and the alpha-subclass as a whole group, ALF968, were used. In a first step, the probes were compared with available sequence information. Databases of 16S rRNA sequences which are accessible to the public [Ribosomal Database Project (RDP), release 7.0, July 1998, Michigan State University, East Lansing, MI, USA; ARB database, release August 1997, Technical University Munich, Germany] were searched for probe-complementary sequences. The probes were found to be still highly specific for the groups of target organisms they were originally designed for.

Fixed cells of 46 members of the *Sphingomonas* cluster including 17 strains previously assigned as *Sphingomonas* sp and additionally *Erythrobacter longus* ATCC 33941^{T} and *Brevundimonas diminuta* DSM 1635^{T} as reference organisms were probed by whole cell hybridization. Results are shown in Table 1. Generally, *Sphingomonas* cells grown as batch cultures in complex media with different contents of organic compounds (nutrient broth and R2A broth) all yielded strong fluorescence signals facilitating microscopic detection (Figure 1). All strains affiliated with the genus *Sphingomonas* except one hybridized with all three probes. *S. asaccharolytica* DSM 10564^{T} showed signals with SPH120 and ALF968 but not with ALF4-1322. Cells of the two *Zymomonas* strains which belong phylogenetically to the *Sphingomonas* cluster also yielded strong signals. *Erythrobacter longus* hybridized, as expected, only with ALF4-1322 but not with SPH120 (Figure 1). The outgroup reference *Brevundimonas diminuta* reacted only with ALF968.

In situ identification of sphingomonads in activated sludge samples

Mixed liquor from six WWTPs was analyzed by FISH for the presence of sphingomonads. Fixed samples of activated sludge were probed with EUB338, ALF968, ALF4-1322, and SPH120. Population sizes determined by cell counting relative to total stains with DAPI are shown in Figure 2. EUB338 counts were around 70% for four municipal plants which treat mainly domestic sewages whereas counts in sludges from the brewery-associated plant Lich, and a pond, Dorf-Güll, were only around 45%. In all six sludges members of the alpha-subclass of the Proteobacteria (probe ALF968) were a significant fraction of the cells which were detected by FISH. Similarly as for EUB338, counts with the specific probes ALF968, ALF4-1322, and SPH120 were again lower for two plants (Dorf-Güll and Lich). Abundances of sphingomonads in the different sludges were between 0.8 and 12%. Counts of ALF4-1322-positive cells were between approximately one-sixth and nearly 50% compared to counts of ALF968-positive cells. Accordingly, the far majority, between 70 and 90% of the ALF4-1322-positive cells also hybridized with the most specific probe SPH120.

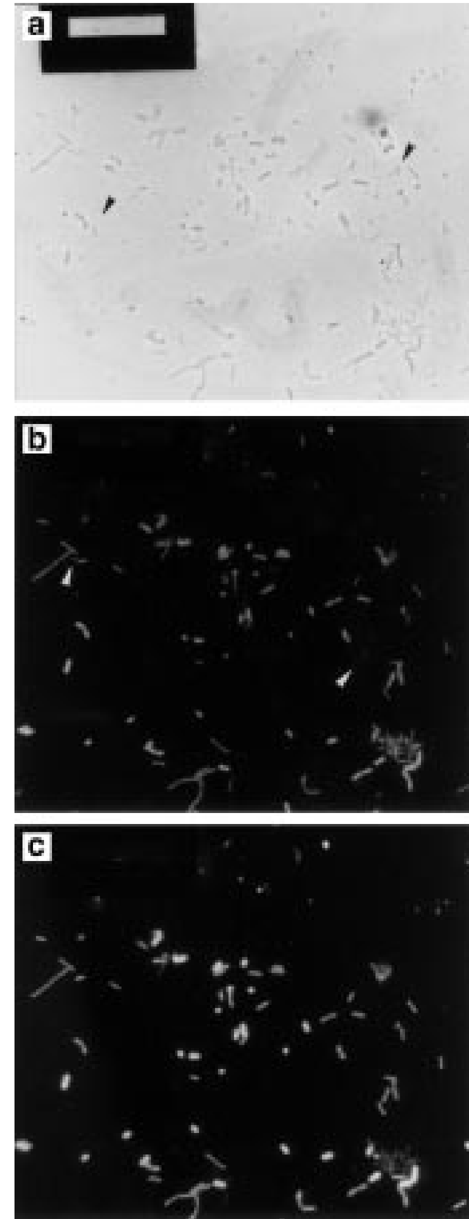

Figure 1 Demonstration of morphodiversity of sphingomonads. Whole cell hybridization of an artificial mixture of five different *Sphingomonas* strains (*S. paucimobilis* NCTC 11030^{T}: short rods, single or pairs; *S. macrogoltabidus* IFO 15033^{T}: long thin rods in chains; *S. terrae* IFO 15098^{T}: single thin rods; *Sphingomonas* sp EDIV: thick rods, single or pairs; *S. natatoria* DSM 3283^{T}: rosette), *Erythrobacter longus* ATCC 33941^{T}, and *Brevundimonas diminuta* DSM 1635^{T}. All three panels represent the same microscopic field. Panel (a) phase contrast micrograph; some *B. diminuta* cells are marked with black arrows. Panels (b) and (c) epifluorescence micrographs show staining results after hybridization with fluoresceine-labeled probe ALF4-1322 (b) and rhodamine-labeled probe SPH120 (c), respectively. Some *E. longus* cells in panel (b) are marked with white arrows. Bar = 20 μm.

The presence of sphingomonads in activated sludges was also compared with respect to spatial distribution in the

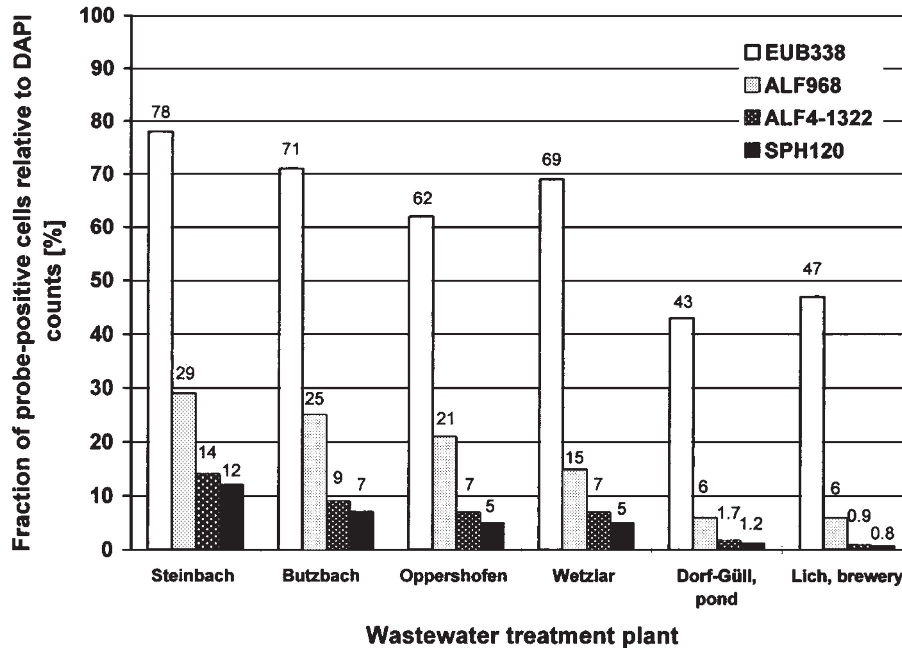

Figure 2 Cell counts of probe-positive cells in six wastewater treatment plants in central Hesse, Germany. The percentage value for each group is noted above each column.

sludge samples. Most sphingomonads were found to be localized inside flocs. In some samples the cells were randomly distributed within the floc material. But there were also samples (WWTPs Steinbach, Butzbach, and Wetzlar) in which 30–50% of the sphingomonads were arranged in aggregates (Figure 3). These aggregates consisted of up to approximately 50 cells which were clustered in variable package densities.

Discussion

Probes ALF4-1322 and SPH120 had been originally tested with only four *Sphingomonas* strains [19]. Whole cell hybridization tests of more than 40 representatives of the alpha-4 subgroup including nearly all described species of the genus *Sphingomonas* confirmed their specificities. ALF4-1322 detects the complete subgroup and the more narrow SPH120 detects all members of the *Sphingomonas* cluster ie also *Zymomonas mobilis* strains which belong to this group according to 16S rRNA sequence data. The strong hybridization signals of cultivated cells demonstrate that FISH could be a useful method for identification of sphingomonads in pure and mixed cultures.

The lack of hybridization of *S. asaccharolytica* DSM 10564^T with probe ALF4-1322 probably results from discrimination by a single mismatch. The 16S rRNA sequence of *S. asaccharolytica* IFO 15499^T contains one base change (A to U) at position 1326 in the target sequence [30], which could be expected to prevent binding of the oligonucleotide. Strain DSM 10564^T is identical to IFO 15499^T and thus no hybridization signal should occur at the applied stringency conditions.

Among the 44 *Sphingomonas* strains cultivated under relatively favorable conditions a wide variety of different morphotypes was observed. Even cells from cultures grown

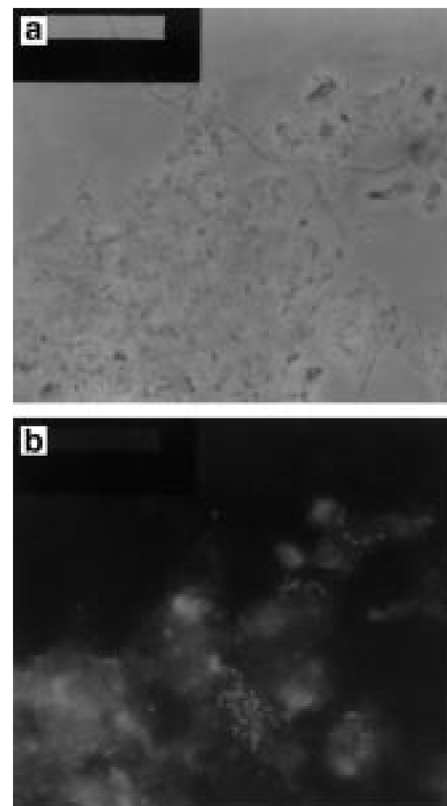

Figure 3 *In situ* identification of sphingomonads in activated sludge. Panel (a), phase contrast picture of a sample from WWTP Wetzlar. Panel (b) epifluorescence picture of the same microscopic field after hybridization with probe SPH120. Bar = 20 μm.

in parallel (Nutrient broth, 30°C, overnight) harvested at similar OD_{600} values (0.48–0.79) exhibited completely different shapes. Rods had different sizes and occurred singly, in pairs or in short chains (Figure 1). However, as a general methodological advantage, FISH allows clear identification independent of variable cell morphology [34]. Therefore, probing enables single cell detection even of atypical morphotypes and within complex matrices like activated sludge samples.

Four activated sludges from one-stage municipal wastewater treatment plants showed typical counts with the bacterial as well as the alpha-proteobacterial probes [19,32,33,36]. In the two other plants lower numbers of cells with a detectable amount of ribosomes indicate less bacterial activity, probably due to lower pollution loads in the aerobic pond and the secondary stage of the brewery plant. Sphingomonads were an abundant group in sludges from all six WWTPs. Between approximately 1 and more than 10% of all DAPI-stained cells could be assigned as members of the *Sphingomonas* cluster. Detection as sphingomonads was confirmed by positive hybridization results of these cells with the more general probes ALF4-1322 and ALF968. Even earlier, Bond *et al* [7] found members of the alpha-4 subgroup in activated sludge from sequencing batch reactors. Moreover, using FISH with probe ALF4-1322, the alpha-4 subgroup was the dominant subgroup among the alpha-Proteobacteria in various activated sludges [19]. The estimated values reported for ten different WWTPs were in the same range as the counted abundances in our study. The extent of diversity of the sphingomonads detected, namely affiliation to distinct species, cannot be estimated from our results but would need eg analysis using probes with more narrow specificities.

The sphingomonads were not distributed randomly among the sludge flocs as expected for evenly mixed samples. Up to 50% of the SPH120-positive cells were clustered in aggregates of various sizes and cell densities. Such clusters were localized mostly within the interior of sludge flocs. However, other sphingomonads in the same sample remained singly or in groups of a few cells only. Overall, sphingomonads appear to be abundant in many activated sludges. Due to their metabolic versatility [30,38], significant involvement of these bacteria in degradation processes in sewages could be suggested.

Members of the genus are known to be able to produce slimes and/or capsules which consist predominantly of polysaccharides [38,40]. Exopolysaccharides as well as the hydrophobicity of outer cell layers due to the presence of sphingolipids could be the basis for cell aggregation of sphingomonads inside activated sludge flocs. Since exopolysaccharides are a significant part of the polymeric extracellular matrix material of flocs [8,12,29], sphingomonads could be involved in processes of floc formation as reported, eg, for *Zoogloea* [8,15]. Continuous production of extracellular polymeric substances is a prerequisite for proper settling of flocs and, consequently, the stability of the activated sludge process in aerobically operated wastewater treatment plants. Thus, the *in situ*-identified sphingomonads are interesting organisms with respect to the question of how distinct microorganisms participate in floc formation processes.

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