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In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes

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Hitherto, few environmental samples have been investigated by a 'full cycle rRNA analysis'. Here the results of *in situ* hybridization experiments with specific rRNA-targeted oligonucleotide probes developed on the basis of new sequences derived from a previously described comparative 16S rRNA analysis of nocardioform actinomycetes in activated sludge are reported. Application of the specific probes enabled identification and discrimination of the distinct populations of nocardioform actinomycetes in activated sludge. One of the specific probes (DLP) detected rod-shaped bacteria which were found in 13 of the 16 investigated sludge samples from various wastewater treatment plants, suggesting their importance in the wastewater treatment process. Another probe (GLP2) hybridized with typically branched filaments of nocardioforms mainly found in samples from enhanced biological phosphorus removal plants, suggesting that these bacteria are involved in sludge foaming. The combination of *in situ* hybridization with fluorescently labelled rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy improved the detection of nocardioform actinomycetes, which often showed only weak signals inside the activated-sludge flocs.

Keywords: fluorescence *in situ* hybridization, 16S rRNA, activated sludge, nocardioform actinomycetes, confocal laser scanning microscopy

INTRODUCTION

Foaming of activated sludge due to an extensive multiplication of filamentous bacteria causes serious separation problems in sewage treatment plants throughout the world (Lemmer & Kroppenstedt, 1984; Blackbeard *et al.*, 1986; Blackall *et al.*, 1991). In addition to *Microthrix parvicella*, a filamentous bacterium belonging to the group of high G+C Gram-positive bacteria (Blackall *et al.*, 1994a), branched, filamentous members of the genera *Gordona*, *Nocardia* and *Rhodococcus* are generally considered to be the cause of foaming in sewage treatment plants (Lechevalier & Lechevalier, 1974; Lechevalier *et al.*, 1976; Blackbeard *et al.*, 1986; Blackall *et al.*, 1988). The reasons for the extensive multiplication of these filamentous bacteria are still unknown (Soddell & Seviour, 1990). Fast and reliable cultivation-independent identification techniques for filamentous bacteria in activated sludge are a prerequisite for the evaluation of their importance in the sewage treatment process and investigation of the problems that can arise. Classical approaches fail to differentiate foam-causing species such as *Gordona amarae*, *Rhodococcus rhodochrous* and *Tsukamurella paurometabolum* due to their variable morphology, staining behaviour and fastidious nature (Foot *et al.*, 1992).

Comparative sequence analysis of 16S rRNA sequences directly retrieved from natural microbial communities represents the most powerful method for describing species composition as both cultured and as yet uncultured micro-organisms can be identified. Sequence

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Abbreviations: CLSM, confocal laser scanning microscopy; EBPR, enhanced biological phosphorus removal; FISH, fluorescence *in situ* hybridization.

Table 1. **Whole-cell hybridization of reference strains with rRNA probes applied to ethanol-fixed cultures after mutanolysin treatment**

Species	Source*	Hybridization with probe†:		
		EUB338	HGC69a	MNP1
Acinetobacter sp. BR-2	MIBI	+	−	−
Corynebacterium variabilis	DSM 20536	+	+	+
Dietzia maris	DSM 43672^T	+	+	+
Escherichia coli	ATCC 25922	+	−	−
Gordona amarae	DSM 43392^T	+/−	+/−	+/−
Gordona rubropertincta	DSM 43197^T	+/−	+/−	+/−
Gordona rubropertincta	DSM 43248	+	+	+
Gordona sputi	DSM 44019	+/−	+/−	+/−
Gordona terrae	DSM 43249^T	+/−	+/−	+/−
Mycobacterium fortuitum	IMMH	−	−	−
Mycobacterium gordonae	IMMH	−	−	−
Nocardia asteroides	IMMH	+/−	+/−	+/−
Rhodococcus equi	ITH	+	+	+
Rhodococcus erythropolis	DSM 43066^T	+/−	+/−	+/−
Rhodococcus erythropolis	DSM 43135^T	+	+	+
Rhodococcus erythropolis FM1	MIBI	+	+	+
Rhodococcus fascians	DSM 20669^T	+/−	+/−	−
Rhodococcus globerulus	DSM 43954^T	+	+	+
Rhodococcus luteus	DSM 43673^T	+	+	+
Rhodococcus marinonascens	DSM 43752^T	+	+	+
Rhodococcus rhodnii	DSM 43336^T	+/−	+/−	+/−
Rhodococcus rhodochrous	DSM 43241^T	+	+	+
Rhodococcus ruber	DSM 43338^T	+	+	+
Rhodococcus sp.	DSM 427	+	+	+
Tsukamurella paurometabolum	DSM 43246	+/−	+/−	−
Tsukamurella paurometabolum	DSM 43274	+/−	+/−	−

* T, Type strain; ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IMMH, Institut für Medizinische Mikrobiologie und Hygiene, Freiburg, Germany; ITH, Institut für Tierhygiene, Freiburg, Germany; MIBI, Mikrobiologisches Institut der Fakultät für Biologie II, Freiburg, Germany.

† +, Bright signal; −, no signal; +/−, partial signal.

information can be used to design rRNA-targeted oligonucleotide probes which enable quantification and analysis of the spatial distribution of these microorganisms in ecosystems by fluorescence *in situ* hybridization (FISH) (Amann *et al.*, 1995).

While whole-cell hybridization of Gram-negative bacteria works well, *in situ* hybridization of some groups of Gram-positive bacteria remains rather difficult. The members of the nocardioform genera *Corynebacterium*, *Dietzia*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Tsukamurella* possess rigid cell envelopes containing mycolic acids with very long aliphatic chains. Consequently, the cell walls of these bacteria are much more difficult to permeabilize, so that probe targets are not accessible to fluorescently labelled oligonucleotide probes. Therefore, methods such as short-time paraformaldehyde fixation (De Los Reyes *et al.*, 1997), enzymic digestion (Hahn *et al.*, 1992) and mild acid hydrolysis (Macnaughton *et al.*, 1994) have been applied to optimize permeabilization of bacteria with mycolic-acid-containing cell walls for fluorescent oligonucleotide probes.

In an earlier study (Schuppler *et al.*, 1995), a molecular approach (Olsen *et al.*, 1986) was used to retrieve new 16S rRNA sequences from nocardioform actinomycetes present in an activated-sludge sample. Based on these sequences, we developed and here describe the use of a set of fluorescently labelled rRNA-targeted oligonucleotide probes to study the presence and abundance of the respective target bacteria in activated sludge from various sewage treatment plants.

METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. Bacteria were cultured as described in the catalogue of strains from the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Nocardioform actinomycetes were cultured in modified Czapek–Dox media (Lechevalier, 1989). Cells were harvested at late exponential phase by centrifu-

Table 2. *In situ* identification of nocardioform actinomycetes in activated sludge from sewage treatment plants in Germany

+, Probe target population detectable; −, probe target population not detectable.

Sewage treatment plant	Hybridization with probe:					
	MNP1	MLP	DLP	GLP1	GLP2	GLP3
Aldersbach	+	−	+	−	−	−
Aretsried	+	−	+	−	+	−
Breisach	−	−	−	−	−	−
Darmstadt*	+	−	−	−	+	−
Dietersheim	+	−	+	−	−	−
Essen-Werden	+	−	+	−	−	−
Großlappen	+	−	+	−	−	−
Hattingen	+	−	+	−	−	−
Hirblingen*	+	−	+	−	−	−
Kraftisried	−	−	−	−	−	−
Marienfelde*	+	−	+	−	+	−
Moosbach	+	−	+	−	−	−
Schönerlinde	+	−	+	−	−	−
Stahnsdorf	+	−	+	−	−	−
Waßmannsdorf*	+	−	+	−	+	−
Wyk	+	−	+	−	−	−

*EBPR plant.

Table 3. Probe sequences, target sites, hybridization temperature for dot-blot hybridization, and formamide concentration in the hybridization buffer required for specific *in situ* hybridization

Probe	Sequence (5′–3′)	Target site*	Hybridization temp. (°C)	Formamide concn (%)
HGC69a†	TATAGTTACCTCCGCCGT	23S, 1901–1918	NU	25
EUB338‡	GCTGCCTCCCGTAGGAGT	16S, 338–355	54	15
MNP1§	TTAGACCCAGTTTCCCAGGCT	16S, 152–172	55	50
MLP‖	AACCCATGCAGGCCGTAGTCC	16S, 182–202	62	ND
DLP‖	CCACCATGCGGCAGGAGCTCA	16S, 182–202	70	40
GLP1‖	ATGCAGTGGAAGGTAATATC	16S, 174–193	50	ND
GLP2‖	AAGGGCAGGTCATATCCGGT	16S, 178–197	58	45
GLP3‖	CCAACCATGCAGTCAGAGGTC	16S, 182–202	58	ND

NU, Not used for dot-blot hybridization; ND, formamide concentrations were not determined for probes MLP, GLP1 and GLP3 as these probes could not be used for FISH.

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

†Roller *et al.* (1994).

‡Stahl & Amann (1991).

§Schuppler *et al.* (1995).

‖Derived from sequences of Schuppler *et al.* (1995) (see Fig. 1).

gation (2 min, 5000 *g*) and washed in PBS (130 mM NaCl, 10 mM sodium phosphate buffer; pH 7·4).

Cell fixation and pre-treatment. Activated-sludge samples were collected from the aeration basins of sewage treatment plants (Table 2) and fixed immediately by addition of ethanol to a final concentration of 50% (v/v). In the laboratory, the activated sludge was centrifuged (2 min, 5000 *g*) and resuspended in PBS/ethanol (1:1, v/v). Gram-positive reference strains were fixed in PBS/ethanol (1:1, v/v) (Roller *et al.*, 1994), whereas Gram-negative reference strains were fixed in paraformaldehyde (4%, w/v, in PBS) and processed as described by Amann *et al.* (1990a). After dehydration, immobilized nocardioform bacteria and activated-sludge samples were pre-treated by adding 10 µl enzyme solution [5000 U mutanolysin ml^{-1} from *Streptomyces globisporus* (Sigma) in 0·1 M potassium phosphate buffer, pH 6·2] and

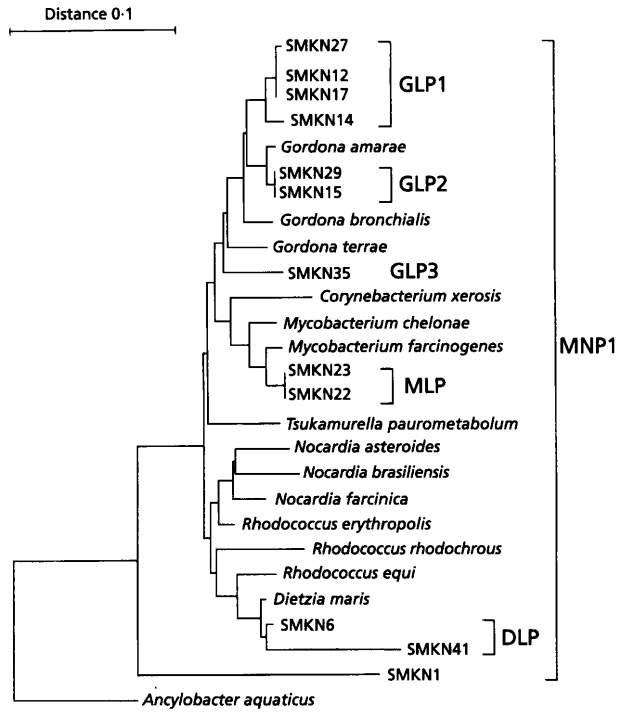

Fig. 1. Specificity of probes MNP1, MLP, DLP, GLP1, GLP2 and GLP3. The dendrogram is based on partial 16S rRNA gene sequences and indicates the phylogenetic relationships among selected Gram-positive bacteria and the clone sequences (SMKN) from a former 16S rRNA sequence analysis of an activated-sludge sample (Schuppler *et al.*, 1995). The scale-bar represents 10% difference in nucleotide sequence.

incubating for 10 min at room temperature. To remove the mutanolysin solution, slides were rinsed with distilled water, air-dried and dehydrated again. Lysozyme treatment was performed according to the protocol of Beimfohr *et al.* (1993).

16S rRNA sequence analysis. Sequence analysis and construction of dendrograms was done as previously described by Schuppler *et al.* (1995).

Oligonucleotide probes. Sequences and the target sites of the oligonucleotide probes used in this study are presented in Table 3. Oligonucleotide probes MLP, DLP, GLP1, GLP2 and GLP3 (Fig. 1) were derived from 16S rRNA sequences described by Schuppler *et al.* (1995). Probes were synthesized with a C6-TFA-aminolinker [6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite; Millipore] at the 5′-end. Labelling of the probes with tetramethylrhodamine-5-isothiocyanate (Molecular Probes) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (Boehringer Mannheim), and purification of the oligonucleotide–dye conjugates were performed as described by Amann *et al.* (1990a). Oligonucleotide probes for dot-blot hybridization were labelled with digoxigenin (DIG oligonucleotide 3′-end labelling kit; Boehringer Mannheim) according to the manufacturer's instructions.

Nucleic acid extraction and dot-blot hybridization. Plasmid DNA preparations of recombinant *Escherichia coli* clones (Schuppler *et al.*, 1995) were dotted on positively charged nylon membranes (Biodyne B; Pall) and immobilized by baking for 2 h at 80 °C. Hybridization with digoxigenin-labelled oligonucleotide probes was done in hybridization tubes (Bachofer). Membranes were pre-hybridized for 1 h at 50 °C in 20 ml hybridization buffer without oligonucleotide probe [5 × SSC, 2% (w/v) blocking reagent (Boehringer Mannheim), 0·1% (w/v) *N*-lauroylsarcosine, 0·2% (w/v) SDS] followed by hybridization with 3 ml hybridization buffer containing 50 pmol labelled oligonucleotide probe for 2 h at the appropriate temperature for each probe. After hybridization, the membranes were washed twice in 20 ml washing buffer [5 × SSC, 0·2% (w/v) SDS] for 15 min at the hybridization temperature. Detection was carried out using anti-DIG antibodies coupled with alkaline phosphatase (DIG Luminescent Detection kit; Boehringer Mannheim) as indicated by the manufacturer. Chemiluminescence was detected by exposure of membranes to X-ray films (Kodak X-Omat AR). Prior to rehybridization, the membranes were stripped by pouring boiling 0·1 × SSC, 0·2% (w/v) SDS over the membranes followed by incubation until the buffer reached room temperature.

***In situ* hybridization.** *In situ* hybridization of fixed materials was carried out at a constant temperature of 46 °C in an isotonically equilibrated humidity chamber according to the method of Amann *et al.* (1990a). On each well of the microscope slide, 8 µl hybridization buffer (0·9 M NaCl; 0·02 M Tris/HCl, pH 7·2; 0·01%, w/v, SDS) and 50 ng probe were applied and incubated for 2–4 h. Stringent hybridization conditions for the different oligonucleotide probes were adjusted by different formamide concentrations in the hybridization buffer as described by Manz *et al.* (1992).

Microscopy and documentation. Slides were examined using a Leitz DMRB microscope (Leica) with filter sets I3 and N2.1. Colour photomicrographs were prepared with Fuji P 1600 colour reversal film. Exposure times were 0·01–0·03 s for phase-contrast photomicrographs and 15–25 s for epifluorescence photomicrographs.

Confocal laser scanning microscopy (CLSM) was done on a Carl Zeiss LSM 410 equipped with an Ar-ion laser (488 nm) and a HeNe laser (543 nm) by recording optical sections. Image processing, depth profiles and three-dimensional reconstructions were performed with the standard software package delivered with the device.

RESULTS

Comparison of different fixation and pre-treatment procedures for permeabilization of nocardioform actinomycetes for whole-cell hybridization

Fixation and hybridization conditions normally used for *in situ* hybridization of Gram-negative bacteria are often unsuitable for Gram-positive bacteria (Roller *et al.*, 1994). This effect can be easily visualized by *in situ* hybridization of a paraformaldehyde-fixed mixed culture of *Acinetobacter* sp. and *Rhodococcus erythropolis* FM1 cells with the eubacteria-specific oligonucleotide probe EUB338. While the Gram-negative *Acinetobacter* sp. cells are intensively stained, the cells of the Gram-positive species *R. erythropolis* give poor and non-homogeneous fluorescence signals (Fig. 2a).

To improve the hybridization efficiency of mycolic-acid-containing nocardioform actinomycetes, we tested the influence of different fixation protocols on cell wall permeability to fluorescently labelled oligonucleotide

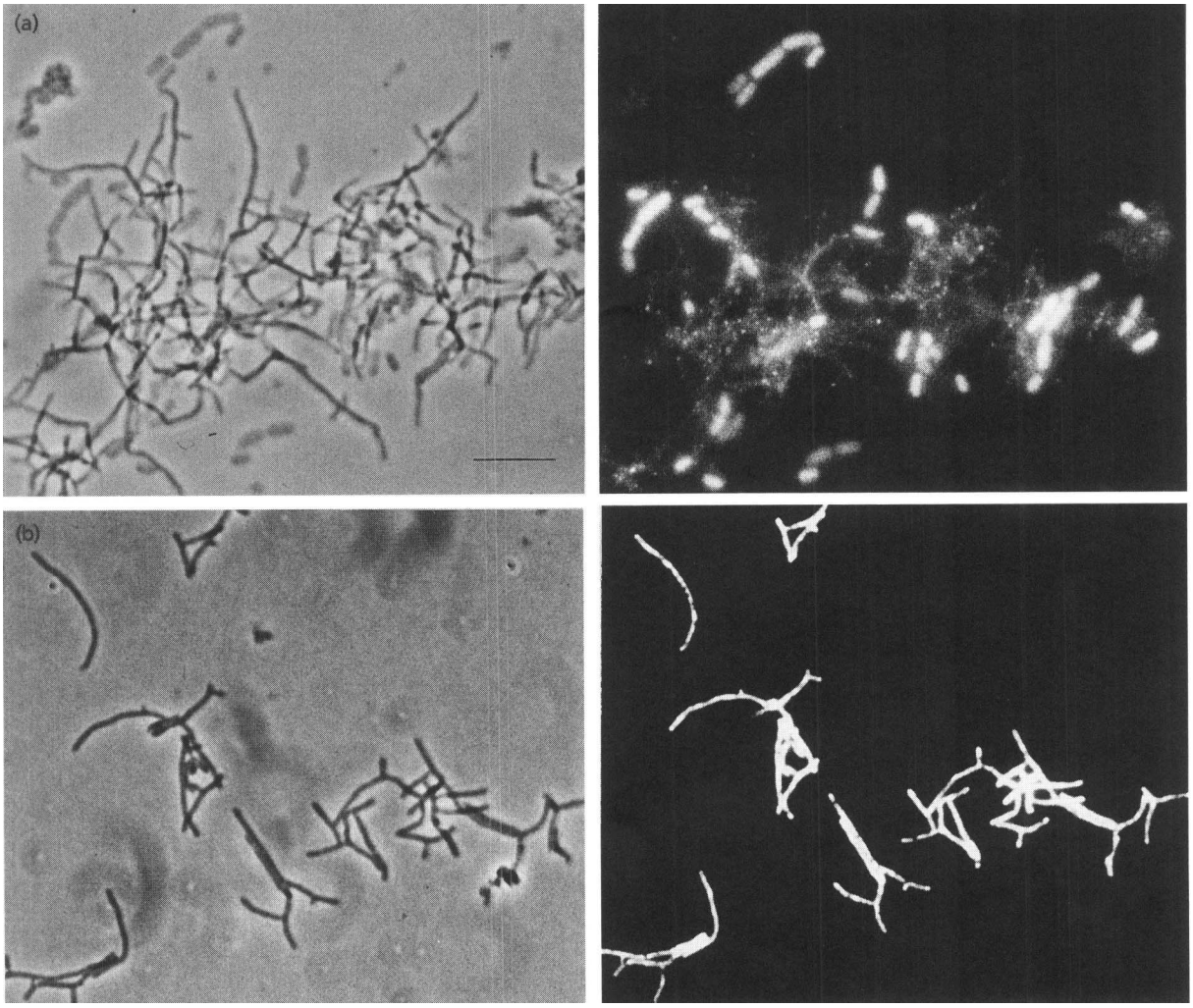

Fig. 2. Fixation and pre-treatment of nocardioform actinomycetes for FISH. For each panel, identical fields were viewed by phase-contrast microscopy (left) and epifluorescence microscopy (right). Bar, 10 μm. (a) Whole-cell hybridization of an artificial mixture of paraformaldehyde-fixed *Acinetobacter* sp. BR-2 and *R. erythropolis* FM1 with fluorescein-labelled probe EUB338. (b) *In situ* hybridization of a pure culture of ethanol/PBS-fixed and mutanolysin-treated *R. erythropolis* FM1 with tetramethylrhodamine-labelled probe MNP1.

probes. For most of the members of the nocardioform actinomycetes analysed, cell fixation with ethanol/PBS (1:1, v/v) (Roller *et al.*, 1994) was more effective (Fig. 2b) than the addition of formaldehyde (DeLong *et al.*, 1989) or glutaraldehyde (Amann *et al.*, 1990b) as fixative. However, after fixation with 50% ethanol, many of our nocardioform actinomycete reference strains still showed little or no fluorescent staining (data not shown). Whereas additional pre-treatment of the ethanol-fixed cells with lysozyme (Beimfohr *et al.*, 1993) led to lysis of many nocardioform actinomycetes, a short incubation with mutanolysin (Assaf & Dick, 1993) further improved probe accessibility to most reference strains while preserving morphological integrity (Table 1). It should be noted that this treatment did not permeabilize members of the genus *Mycobacterium* for *in situ* hybridization (Table 1) and completely lysed almost all of the Gram-negative bacteria strains analysed (data not shown). Extension of mutanolysin incubation times to > 10 min was not found to be suitable for *in situ* hybridization as it increased the number of damaged nocardioform target cells. Treatment of ethanol-fixed nocardioform actinomycetes with organic solvents (acetone, chloroform, diethyl ether, n-hexane, methanol and toluol, and combinations of these solvents) was in general found to be ineffective in further permeabilizing nocardioform cell walls for *in situ* hybridization.

In situ identification of nocardioform actinomycetes in activated sludge from the Berlin-Marienfelde sewage treatment plant

In situ hybridization using the nocardioform-specific oligonucleotide probe MNP1 (Fig. 1) was performed to identify populations of nocardioform actinomycetes in the activated sludge from the Berlin-Marienfelde sewage

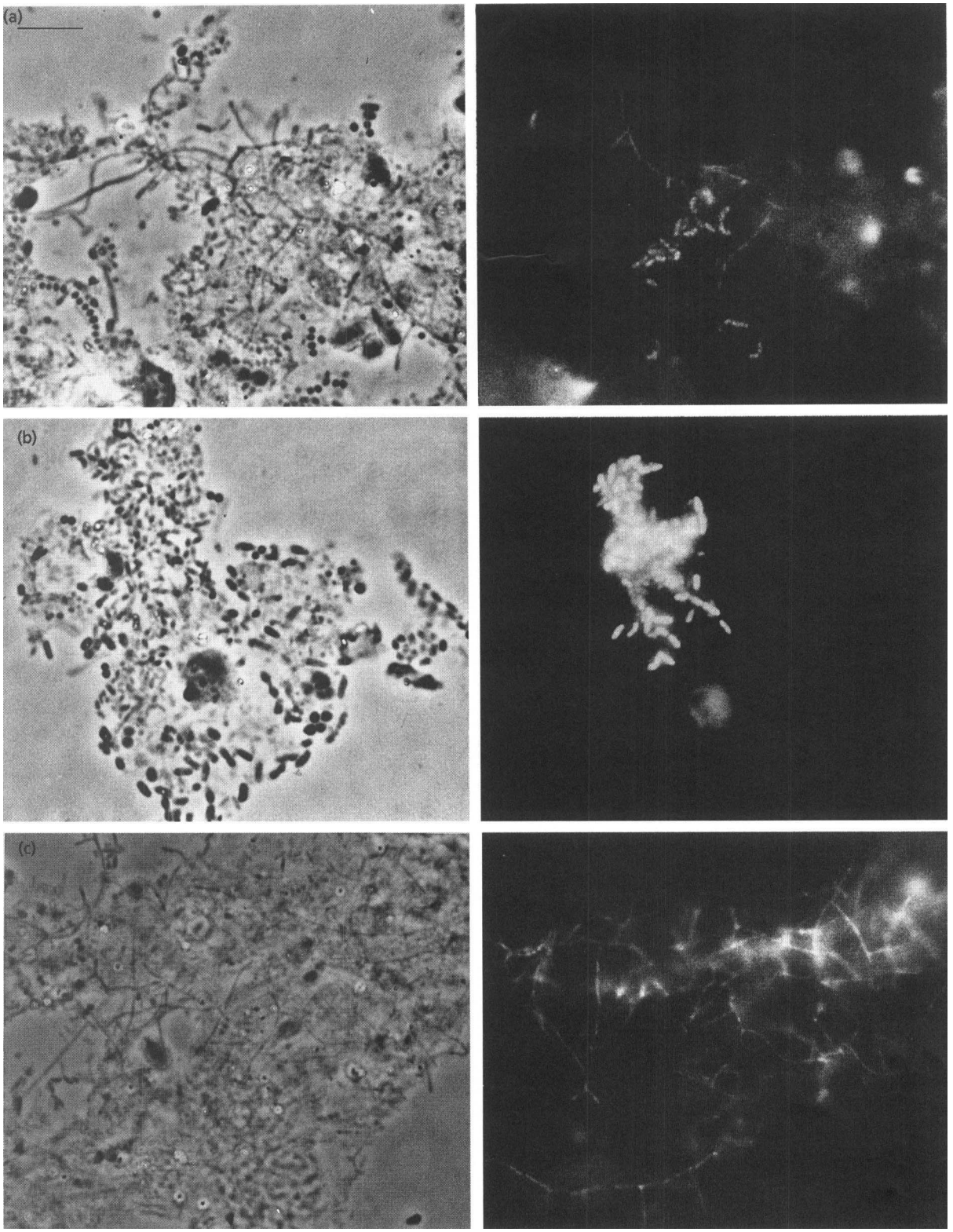

Fig. 3. Whole-cell hybridization of nocardioform actinomycetes in an activated-sludge sample from the Berlin-Marienfelde wastewater treatment plant. For each panel, identical fields were viewed by phase-contrast microscopy (left) and epifluorescence microscopy (right). Bar, 10 μm. (a) *In situ* identification of nocardioform bacteria with fluorescein-labelled probe MNP1. (b) *In situ* identification of short rod-shaped nocardioform actinomycetes with tetramethylrhodamine-labelled probe DLP. (c) *In situ* identification of typical branched filaments of nocardioform actinomycetes with tetramethylrhodamine-labelled probe GLP2.

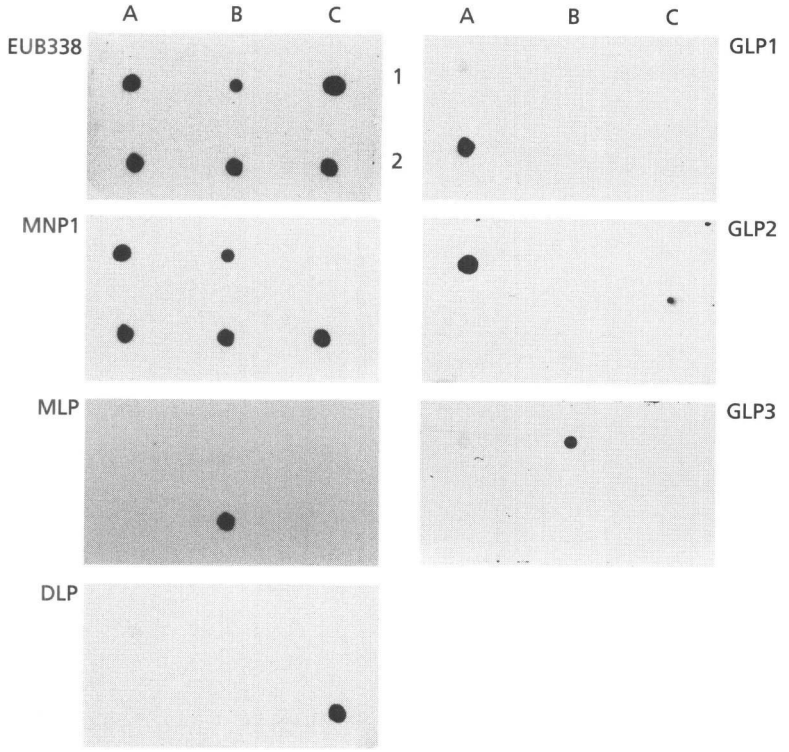

Fig. 4. Dot-blot analysis of probe specificity. Recombinant plasmid DNA from one clone of each of the five groups of nocardioform clones, and from one clone containing a non-nocardioform 16S rDNA (Schuppler *et al.*, 1995), was hybridized with the oligonucleotide probes EUB338, MNP1, MLP, DLP, GLP1, GLP2 and GLP3. Arrangement of plasmid DNA on the membrane: A1, clone SMKN15; A2, clone SMKN12; B1, clone SMKN35; B2, clone SMKN23; C1, clone SMK196; C2, clone SMKN41. For phylogenetic affiliation of the dotted 16S rRNA sequences, see Fig. 1. All probes hybridized specifically to their target clones.

treatment plant. For this experiment, we used fixed material from the identical sludge sample which had been used in a previously described molecular characterization of nocardioform actinomycetes by 16S rRNA analysis (Schuppler *et al.*, 1995). *In situ* hybridization with probe MNP1 resulted in the detection of two populations with different morphologies. One morphotype represented typical branched filaments of nocardioform actinomycetes, whereas the other morphotype comprised short irregular rods (Fig. 3a). Both populations simultaneously hybridized with probe HGC69a (Roller *et al.*, 1994), confirming that the bacteria belong to the group of actinomycetes and their relatives (data not shown).

Specificities of probes MLP, DLP, GLP1, GLP2 and GLP3 (Fig. 1) derived from nocardioform-actinomycete-affiliated sequences described by Schuppler *et al.* (1995) were tested by dot-blot hybridization (Fig. 4). While the eubacteria-specific probe EUB338 hybridized to all of the plasmid DNA preparations tested, probe MNP1 bound exclusively to plasmid DNA from clones containing nocardioform 16S rDNA, confirming the specificity of probe MNP1. As expected, the five specific oligonucleotide probes MLP, DLP, GLP1, GLP2 and GLP3 hybridized exclusively to their respective target 16S rDNA.

The application of these specific probes for *in situ* hybridization of activated sludge from Berlin-Marienfelde enabled a further discrimination of the different populations of nocardioform actinomycetes detected by probe MNP1. Whereas probe DLP specifically detected the short irregular rods, probe GLP2 identified the branched filaments in the activated sludge (Fig. 3). All bacteria identified with either probe DLP or probe GLP2 were simultaneously detectable *in situ* with probe MNP1. According to a recent database check using the ARB program package, all but one non-target bacteria for probes DLP and GLP2, which possess the target region of probe MNP1, have at least two mismatches to the respective probe target regions demonstrating the desired probe specificities (Table 4). Although *Dietzia maris* has only one mismatch to the target region of probe DLP, whole-cell hybridization experiments demonstrated that it is not detected by probe DLP under stringent hybridization conditions (data not shown).

In spite of the fact that all probes were designed on the basis of nocardioform sequences from the previously described clone library (Schuppler *et al.*, 1995), probes MLP, GLP1 and GLP3 failed to detect bacterial populations in the activated-sludge sample from the Berlin-Marienfelde sewage treatment plant.

In situ detection of nocardioform actinomycetes in activated sludge from different wastewater treatment plants

To address the question of whether distinct populations of nocardioform actinomycetes in the Berlin-Marienfelde wastewater treatment plant are characteristic for plants removing phosphorus biologically (EBPR plants), fixed activated sludge from plants with and without EBPR (Table 2) was analysed. Initially, all sludge samples were simultaneously hybridized with probes

Table 4. Difference alignment of the 16S rRNA target regions of probes DLP and GLP2

Organism	Target sequence of probe DLP	Target region of probe MNP1*
	UGAGCUCCUGCCGCAUGGUGG	
Brachybacterium faecium	------U--------------	–
Dietzia maris	---A-----------------	+
Mycobacterium chitae	G----------U---------	–
Propionibacterium acnes	G----------U---------	–
Rhodococcus equi	----------U-------C--	+
Organism	**Target sequence of probe GLP2**	**Target region of probe MNP1***
	ACCGGAUAUGACCUGCCCUU	
Gordona amarae	----------------U-C-	+
Nocardia carnea	----------------AG--	+
Rathayibacter rathayi	------------------CG	–

* See also Table 1.

MNP1 and HGC69a. In all samples, cells detectable with probe MNP1 were also identified with probe HGC69a, confirming that they were members of the group of Gram-positive bacteria with a high DNA G+C content.

Sludge samples containing nocardioform populations detected by probe MNP1 were further analysed by hybridization with the specific probes DLP, GLP1, GLP2, GLP3 and MLP (Table 3). These specific probes were always applied simultaneously with probe MNP1. In 13 of the 16 analysed sludge samples, probe DLP identified short irregular rods morphologically identical to those detected in the Berlin-Marienfelde wastewater treatment plant (Fig. 5a, b). These bacteria, which also hybridized with probe MNP1, were particularly abundant in activated sludge obtained from the Hirblingen and Aldersbach plants. In samples from Aretsried, Darmstadt and Waßmannsdorf, we found branched filaments of nocardioform actinomycetes which bound probe GLP2 and probe MNP1. Corresponding to the results from Berlin-Marienfelde, no bacteria were detected with the specific probes MLP, GLP1 and GLP3 in all sludge samples analysed.

In situ analysis of nocardioform actinomycetes by CLSM

The use of CLSM enabled improved detection of bacteria inside sludge flocs by eliminating unfocused fluorescence. In addition, digital contrast enhancement and elimination of sections without information facilitated visualization of bacteria that gave only weak signals inside the sludge flocs (Fig. 5b, c). However, not all nocardioform bacteria detectable by phase-contrast gave bright signals after *in situ* hybridization. It was often observed that filamentous bacteria showed an irregular distribution of fluorescence over the filaments (Fig. 5c).

DISCUSSION

In situ hybridization with fluorescent rRNA-targeted oligonucleotide probes represents the most powerful technique for the direct *in situ* identification of single bacterial cells in complex microbial ecosystems. The application of *in situ* hybridization has enabled investigation of the structure and dynamics of microbial communities in activated sludge (e.g. Wagner *et al.*, 1993; Manz *et al.*, 1994; Wagner *et al.*, 1994b, c, 1995; Mobarry *et al.*, 1996; De Los Reyes *et al.*, 1997).

On the basis of new nocardioform 16S rRNA sequences described by Schuppler *et al.* (1995), we designed specific oligonucleotide probes to identify the respective bacteria in activated sludge. *In situ* hybridization with probes DLP and GLP2 allowed the assignment of the two nocardioform populations that were initially detected by probe MNP1 to the respective sequence clusters of the comparative 16S rRNA analysis. Probe DLP was designed on the basis of clone sequences SMKN6 and SMKN41 (Schuppler *et al.*, 1995). The high degree of sequence similarity to 16S rDNA of *D. maris* (Rainey *et al.*, 1995a, b) suggested that the short irregular rods are closely related to this species originally isolated from soil (Nesterenko *et al.*, 1982). Probe GLP2 is derived from clone sequences SMKN15 and SMKN29 (Schuppler *et al.*, 1995), indicating that the bacteria identified by this probe represent close relatives of *G. amarae* (Blackall *et al.*, 1994b; Ruimy *et al.*, 1994), a species frequently found in activated sludge (Lechevalier & Lechevalier, 1974). The *in situ* identification of these bacteria in activated sludge by FISH closed the gap to a 'full cycle rRNA analysis' (Amann *et al.*, 1995). Unfortunately, the destructive effect of the mutanolysin treatment for most of the Gram-negative bacteria tested made it impossible to determine precisely the relative proportion of *in situ* identified nocardioform actinomycetes.

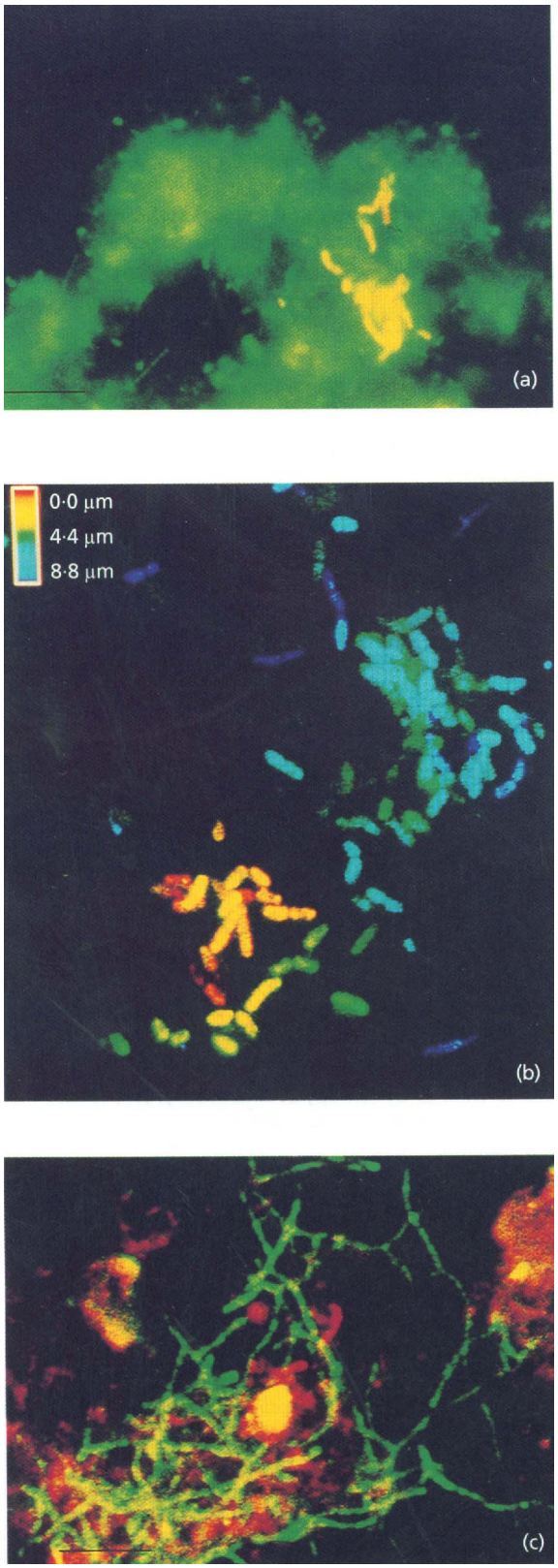

Fig. 5. (a) Simultaneous *in situ* hybridization of activated-sludge samples obtained from the wastewater treatment plant Essen-Werden with fluorescein-labelled probe EUB338 and tetramethylrhodamine-labelled probe DLP. Bar, 10 μm. High background fluorescence obtained with the bacterial probe is mainly caused by Gram-negative cell lysis due to the fixation and pre-treatment procedures applied. (b) CLSM analysis of *in situ* hybridization preparations from ethanol/PBS-fixed activated sludge from the Hirblingen wastewater treatment plant with tetramethylrhodamine-labelled probe DLP. The depth profile shows the three-dimensional location of the bacteria indicated by different colours. (c) CLSM analysis of *in situ* hybridization preparations from ethanol/PBS-fixed activated sludge from the Berlin-Marienfelde wastewater treatment plant with fluorescein-labelled probe MNP1. Bar, 10 μm (also applies to b). Autofluorescence of the activated-sludge preparations was minimized by selecting suitable optical sections and digital contrast enhancement procedures.

The observation that no bacteria were detected with probes MLP, GLP1 and GLP3 in the activated-sludge material could be due to several reasons. Firstly, the cloned 16S rDNA which was used to design these probes could have originated from free DNA or DNA from dead cells. Secondly, the fixation and hybridization conditions used in this study were not suitable for all species of nocardioform actinomycetes (Table 1). The cell walls of *Mycobacterium* species contain mycolic acids with extremely long aliphatic chains and are consequently not permeable to the fluorescently labelled probes, even after treatment with mutanolysin. As the target 16S rDNA sequences for probe MLP (SMKN22 and SMKN23) are highly similar to those of the genus *Mycobacterium* (Fig. 1), it is not surprising that no target bacteria for probe MLP were detectable by *in situ* hybridization.

The target molecule for the fluorescent oligonucleotide probes is rRNA. Consequently, an insufficient number of ribosomes per cell due to reduced metabolic activity could be another reason for the failure of *in situ* detection with probes MLP, GLP1 and GLP3. Wagner *et al.* (1993) found that members of the family *Enterobacteriaceae* were not detectable by *in situ* hybridization if the activated sludge was fixed immediately after sampling. However, if the sludge sample was incubated with nutrients prior to fixation, the respective bacteria gave bright signals. In addition, the three-dimensional arrangement of the rRNA molecule results in blockage of specific regions for *in situ* hybridization, preventing the detection of bacteria even though they possess a sufficient number of ribosomes and show good permeability for oligonucleotide probes (Amann *et al.*, 1995).

The investigation of various wastewater treatment plants by *in situ* hybridization of activated-sludge samples with oligonucleotide probe MNP1 indicated that nocardioform actinomycetes are widely distributed. Populations of nocardioform actinomycetes were detected in nearly all plants investigated, an observation which correlates with the results of Bark *et al.* (1993). Hybridization with probe DLP showed a widespread occurrence of short irregular rods, sometimes in very high numbers, in activated sludge. This observation suggests that these bacteria play an important role in the

wastewater treatment process. However, their detection in high numbers in wastewater treatment plants both with (e.g. Hirblingen) and without (e.g. Aldersbach) EBPR does not enable any conclusions on their possible role in the biological removal of phosphorus.

In contrast to the short irregular rods, the typically branched filaments of nocardioform actinomycetes identified *in situ* by probe GLP2 were found only occasionally. Some sewage treatment plants contained filamentous forms of nocardioform actinomycetes which were not identified by probe GLP2, suggesting the existence of different populations of bacteria that share the same gross morphology. Interestingly, most sludge samples that contained populations of branched filaments were derived from wastewater treatment plants with EBPR (Berlin-Marienfelde, Darmstadt, Essen-Werden, Waßmannsdorf). It is well-established that filamentous forms of nocardioform actinomycetes absorb and store polyphosphates (Schön, 1994) and since plants of the EBPR type are particularly prone to foaming problems, it could be proposed that branched filamentous forms of nocardioform actinomycetes are involved in both processes, i.e. EBPR and sludge foaming. This hypothesis is also supported by recent findings that high G+C Gram-positive bacteria represent an important, polyphosphate-accumulating part of the microbial community in EBPR plants which increases in number after anaerobic acetate dosage (Wagner *et al.*, 1994a).

In this study, the use of CLSM enabled improved detection of nocardioform actinomycetes in activated sludge by *in situ* hybridization with fluorescently labelled oligonucleotide probes, even inside sludge flocs. Investigation of their spatial distribution showed that physiologically active bacteria (indicated by a high cellular rRNA content) were not restricted to the outer area of sludge flocs. This suggests that bacteria inside the sludge flocs are provided with sufficient nutrients for activity.

In situ hybridization of Gram-positive filamentous bacteria often resulted in an irregular distribution of fluorescence signals over the whole filaments. While some parts gave bright signals, other parts of the same filament exhibited no fluorescence. The borderlines of single cells in the filaments are invisible. Consequently, it remains unresolved whether different parts of the filaments belong to the same cell or not. A possible explanation for the staining pattern observed would be that parts showing no signals represent dead cells or at least cells with a low metabolic activity. Variations in permeability of the cell envelope within the filament is another possible explanation of this phenomenon.

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