***In situ* identification of Legionellaceae using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy**

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Bacteria of the family Legionellaceae form a monophyletic group within the γ -subclass of Proteobacteria. Based on comparative sequence analysis we constructed two oligonucleotide probes complementary to regions of 16S rRNA characteristic for Legionellaceae. Probe specificities were tested by whole-cell or dot-blot hybridization against 14 serogroups of Legionella pneumophila, 22 different Legionella spp. and 72 non-legionellae reference strains. Using optimized conditions both probes hybridized to all tested strains of L. pneumophila. Probes LEG226 and LEG705 hybridized to 71% and 90% of the Legionella species tested, respectively. With the exception of Methylobacterium alba none of the non-target strains showed complete sequence homology within the target molecule. In a preliminary evaluation the results of classical techniques employing selective media, immunofluorescence and the probe assay were in good accordance for routine environmental and clinical isolates. L. pneumophila suspended in drinking water at approximately 10^3 – 10^4 c.f.u. ml⁻¹ could be rapidly detected by a combination of membrane filtration on polycarbonate filters and whole-cell hybridization. Even after incubation for 1 year a proportion of the released cells was still detectable. In situ hybridization also facilitated visualization of Legionella spp. cells in model biofilms. A combination of in situ hybridization and confocal laser scanning microscopy (CLSM) was used to analyse the three-dimensional arrangement of L. pneumophila within cells of the ciliated protozoan Tetrahymena pyriformis. Whole-cell probing with 16S rRNA-targeted oligonucleotides could, in the future, complement established techniques like immunofluorescence and PCR in ecological and epidemiological studies of Legionellaceae.

Keywords: *in situ* hybridization, rRNA-targeted oligonucleotide probes, Legionellaceae, confocal laser scanning microscopy

INTRODUCTION

Legionellaceae are ubiquitous environmental microorganisms which can cause severe illnesses including 'Legionnaires' disease' (Brenner *et al.*, 1979). They are found both in natural and man-made environments, such as cooling-towers (Dondero *et al.*, 1980; States *et al.*, 1990; Colbourne & Dennis, 1989). Some 39 species of the genus *Legionella* (Dennis *et al.*, 1993) and 14 distinct

serogroups of the species *Legionella pneumophila* have been described to date. These form a monophyletic group within the γ -subclass of Proteobacteria (Schleifer & Ludwig, 1989; Ludwig & Stackebrandt, 1983; Fry *et al.*, 1991b).

Nosocomial legionellosis has been frequently related to contaminated potable water supplies. However, isolation and cultural recovery of *L. pneumophila* on selective media is often hampered by the presence of faster growing organisms and the ability of Legionellaceae to form viable but nonculturable cells (Hussong *et al.*, 1987; Paszko-Kolva *et al.*, 1992). Cultivation of *L. pneumophila* has

Abbreviations: CLSM, confocal laser scanning microscopy; TRITC, tetramethylrhodamine-5-isothiocyanate.

proven difficult, especially after exposure to biocides and disinfecting agents like hypochlorite (England *et al.*, 1982; Bej *et al.*, 1991).

Legionellae are able to infect free-living amoebae and ciliated protozoa *in vitro* (Fields *et al.*, 1984) as well as in natural environments (Barbaree *et al.*, 1986; Winn, 1988). Intracellular growth of *L. pneumophila* in trophozoites of a variety of amoebae has been shown under laboratory conditions (Kurtz *et al.*, 1982). Consequently, the amoebae species naturally occurring in water could serve as a sort of intermediate host, significantly enhancing the survival of *Legionella* spp. in oligotrophic aquatic habitats. Furthermore, a specific association with free-living amoebae has been used to isolate *Legionellaceae* from clinical and environmental samples (Fields *et al.*, 1989; Rowbotham, 1983; Fallon & Rowbotham, 1990; Fry *et al.*, 1991a).

Due to slow growth and lack of suitable phenotypic tests, identification of *Legionella* spp. by classical techniques remains difficult. To circumvent these limitations, antibody techniques (Edelstein *et al.*, 1985; Gosting *et al.*, 1984), DNA hybridization assays (Grimont *et al.*, 1985), commercial DNA probes complementary to 16S rRNA (Gen-Probe) as well as PCR assays (Mahbubani *et al.*, 1990; Koide *et al.*, 1993; Starnbach *et al.*, 1989) have been developed. The target is in most cases the clinically important species *L. pneumophila*.

Fluorescently labelled, rRNA-targeted oligonucleotide probes are a newly available alternative for *in situ* detection and identification of individual microbial cells (DeLong *et al.*, 1989; Amann *et al.*, 1990a, b; Manz *et al.*, 1993; Wagner *et al.*, 1993). They have successfully been used for the identification of hitherto uncultured bacterial endosymbionts in protozoa, e.g. intranuclear symbionts of the genus *Holospira* spp. in the ciliate *Paramecium caudatum* (Amann *et al.*, 1991) and the legionella-related parasite *Sarcobium lyticum* in small amoebae (Springer *et al.*, 1992). The development and application of oligonucleotide probes specific for the family *Legionellaceae* should not only facilitate determinative studies but also yield tools for investigations on the spatial and temporal distribution of this important group of micro-organisms.

METHODS

Strains and cultivation. Bacterial strains and sources used in this study are listed in Tables 1 and 2. *Legionella* strains were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA) or are held in the culture collection of the *Legionella* reference unit of the Swedish Institute for Infectious Disease Control (SMI, Stockholm, Sweden). *Legionella* strains were cultured on BCYE agar at 37 °C for 2–3 d. Non-legionellae reference strains were cultured following the recommendations of the catalogue of the ATCC. Isolates from tap water samples were cultivated on yeast-peptone agar (yeast extract, 0.6%; peptone, 0.3%; agar, 1.5%, w/v).

Cell fixation. Legionellae were harvested from plates, carefully suspended in sterile PBS at pH 7.4 (PBS: 130 mM NaCl, 10 mM Na₂HPO₄/NaH₂PO₄) and fixed for at least 3 h by adding 3 vols 4% (w/v) paraformaldehyde solution. After washing once with PBS cells were stored in a 1:1 mixture of PBS and 96% (v/v) ethanol at –20 °C. Fixed cells were spotted on precleaned,

gelatin coated [0.01% KCr(SO₄)₂, 0.1% gelatin] microscopic slides (Paul Marienfeld KG, Bad Mergentheim, Germany), air-dried and dehydrated in 50, 80 and 96% (v/v) ethanol (3 min each).

Oligonucleotide probes. The two probes LEG226 and LEG705 have been developed based on a comparative analysis of about 30 aligned 16S rRNA sequences of *Legionellaceae* (Fry *et al.*, 1991b) as described by Amann *et al.* (1990a) and Stahl & Amann (1991), and are complementary to regions of the 16S rRNA of most members of the family *Legionellaceae*. The probe EUB338, complementary to a region of the 16S rRNA conserved in the domain *Bacteria* (Amann *et al.*, 1990b), was used as a positive control. Probe ALF1b, complementary to members of the α -subclass of *Proteobacteria* (Manz *et al.*, 1992), was used for biofilm studies. Probe sequences and target positions are listed in Table 3.

Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] at the 5'-end (MWG Biotech). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes) or 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS, Boehringer Mannheim) was performed as described previously (Amann *et al.*, 1990a). For dot-blot hybridizations the probes were labelled with digoxigenin following the protocols of Zarda *et al.* (1991).

Nucleic acid extraction and dot-blot hybridization. For isolation of nucleic acids from *Legionella* spp., cells were transferred into 200 μ l lysozyme solution (2 mg ml⁻¹), carefully mixed and incubated for 15 min at 37 °C. For cell lysis 200 μ l guanidium thiocyanate buffer (5 M guanidine thiocyanate, 0.1 M EDTA, 0.5%, v/v, N-lauroylsarcosine, pH 8.0) was added. After precipitation of proteins with 150 μ l 2.5 M ammonium acetate (pH 4.5) 550 μ l chloroform/isoamylalcohol (24:1, v/v) was added. After vigorous mixing for 30 s and centrifugation (15 min, 14000 g, 5 min), the aqueous phase was collected and the nucleic acids precipitated with 2 vols ice-cold ethanol (30 min, –20 °C). The mixture was centrifuged (15 min, 14000 g) and the pellet was dried and resuspended in double-distilled water. Nucleic acid isolation from reference strains, immobilization of nucleic acids on nylon membranes and probing with digoxigenin-labelled oligonucleotides were done as described previously (Manz *et al.*, 1992).

Whole-cell hybridization. Whole-cell hybridizations on glass slides were performed as described by Manz *et al.* (1992). For evaluation of the combination of whole-cell hybridization and membrane filtration 1000 ml potable water was inoculated with approximately 10⁴ c.f.u. ml⁻¹ *L. pneumophila* and aliquots of 50 ml were filtered through a 13-mm-diameter, 0.2- μ m-pore-size polycarbonate filter using a 50 ml plastic syringe. Filters were then overlaid with 4% (w/v) paraformaldehyde solution for 30 min, washed twice with 10 ml PBS, and then dehydrated through a 50, 80 and 96% (v/v) ethanol series. A 100 μ l aliquot of hybridization buffer (20%, v/v, formamide, 0.9 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.6) was applied to a glass slide and a single filter was placed on it. For whole-cell hybridization aliquots of 20 μ l hybridization buffer containing 100 ng of the labelled probe were spotted onto the filters and incubated for 1.5 h at 46 °C in a moisture chamber. The filters were removed gently from the slide, transferred into plastic jars filled with washing buffer at pH 7.2 (20 mM Tris/HCl, 180 mM NaCl, 0.01% SDS) and incubated for 15 min at 46 °C. Finally, the filters were removed from the jars, washed once with distilled water and mounted with Citifluor.

In an additional experiment *L. pneumophila* serogroup 1 strain 56/1 (SMI culture collection) was inoculated into 500 ml

Table 1. Reference strains used for dot-blot analysis

Name	Origin*
<i>Acinetobacter calcoaceticus</i>	LMG 1046 ^T
<i>Aeromonas hydrophila</i>	WS 1406
<i>Agrobacterium tumefaciens</i>	ATCC 23308 ^T
<i>Alcaligenes eutrophus</i>	DSM 531 ^T
<i>Alcaligenes faecalis</i>	ATCC 8750 ^T
<i>Alteromonas putrefaciens</i>	DSM 50426
<i>Aquaspirillum metamorphum</i>	DSM 1837 ^T
<i>Azospirillum amazonense</i>	DSM 2787 ^T
<i>Azospirillum brasilense</i>	DSM 1690 ^T
<i>Azospirillum halopraeferens</i>	DSM 3675 ^T
<i>Bacillus cereus</i>	DSM 31 ^T
<i>Bacillus subtilis</i>	ATCC 6633
<i>Brevibacterium ketoglutamicum</i>	DSM 20165
<i>Burkholderia cepacia</i>	DSM 50181
<i>Chromobacterium violaceum</i>	DSM 30191 ^T
<i>Clostridium acetobutylicum</i>	NCIMB 8052 ^T
<i>Clostridium tercorarium</i>	NCIMB 11754 ^T
<i>Comamonas testosteroni</i>	LMG 1800 ^T
<i>Corynebacterium glutamicum</i>	DSM 20300 ^T
<i>Cytophaga hutchinsonii</i>	LMG 10844
<i>Cytophaga johnsonae</i>	LMG 1342
<i>Enterobacter aerogenes</i>	WS 1292
<i>Enterobacter cloacae</i>	WS 1293
<i>Enterococcus cecorum</i>	LMG 11741 ^T
<i>Enterococcus dispar</i>	DSM 6630 ^T
<i>Enterococcus faecalis</i>	DSM 20478 ^T
<i>Enterococcus faecium</i>	DSM 20477 ^T
<i>Enterococcus gallinarum</i>	LMG 11207 ^T
<i>Enterococcus hirae</i>	LMG 6399 ^T
<i>Enterococcus malodoratus</i>	LMG 10747 ^T
<i>Enterococcus mundtii</i>	LMG 10748 ^T
<i>Enterococcus saccharolyticus</i>	LMG 11427 ^T
<i>Erwinia carotovora</i>	WS 1394
<i>Escherichia coli</i>	DSM 30083 ^T
<i>Flavobacterium breve</i>	LMG 4011 ^T
<i>Flavobacterium ferrugineum</i>	LMG 4021 ^T
<i>Flavobacterium gleum</i>	LMG 8334
<i>Flavobacterium indologenes</i>	LMG 8336
<i>Flavobacterium odoratum</i>	LMG 4028
<i>Flavobacterium uliginosum</i>	LMG 3809 ^T
<i>Haliscomenobacter hydrossis</i>	DSM 1100
<i>Lactobacillus casei</i>	LMG 9091
<i>Lactococcus cremoris</i>	DSM 20069 ^T
<i>Lactococcus lactis</i>	DSM 20481 ^T
<i>Leuconthrix mucor</i>	DSM 2157 ^T
<i>Magnetospirillum gryphiswaldense</i>	DSM 6361 ^T
<i>Micrococcus luteus</i>	CCM 169 ^T
<i>Moraxella anatispestifer</i>	LMG 11602
<i>Myxococcus fulvus</i>	GBF Mx-f2
<i>Myxococcus virescens</i>	GBF Mx-v4
<i>Nocardioideus simplex</i>	DSM 20130 ^T
<i>Paracoccus denitrificans</i>	DSM 65 ^T
<i>Pectinatus frisingensis</i>	DSM 20465
<i>Proteus vulgaris</i>	WS 1356
<i>Pseudomonas alcaligenes</i>	DSM 50342 ^T
<i>Pseudomonas fluorescens</i>	DSM 50090
<i>Pseudomonas pseudoalcaligenes</i>	LMG 1225 ^T

<i>Pseudomonas putida</i>	DSM 291 ^T
<i>Pseudomonas stutzeri</i>	DSM 50227
<i>Rhodobacter capsulatus</i>	DSM 1710 ^T
<i>Rhodococcus rhodochrous</i>	DSM 43008
<i>Rhodospirillum rubrum</i>	DSM 107
<i>Sapropirilla grandis</i>	LMG 10407 ^T
<i>Serratia marcescens</i>	WS 1359
<i>Sphaerotilus natans</i>	ATCC 13338 ^T
<i>Sphingobacterium mizutae</i>	LMG 8340 ^T
<i>Sphingobacterium spiritivorum</i>	LMG 8347 ^T
<i>Staphylococcus aureus</i>	DSM 20231 ^T
<i>Streptococcus salivarius</i>	DSM 20560 ^T
<i>Thiobacillus acidophilus</i>	DSM 700 ^T
<i>Weeksellia virosa</i>	LMG 8349
<i>Weeksellia zoobellum</i>	LMG 8352

*T, Type strain; ATCC, American Type Culture Collection, Rockville, MD, USA; CCM, Czechoslovak Collection of Microorganisms, Brno, CSFR; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; NCIMB, The National Collections of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, UK; WS, Collection of the Institut für Mikrobiologie, Forschungszentrum für Milch- und Lebensmittel, Technical University Munich, Freising Weihenstephan, FRG.

autoclaved, filter-sterilized drinking water to achieve an initial concentration of 5×10^3 c.f.u. ml⁻¹. After 1 year of incubation at 30 °C cells were harvested by centrifugation (5000 g, 20 min), resuspended in 10 ml autoclaved, filter-sterilized drinking water, fixed for 3 h with 4% (w/v) paraformaldehyde solution and hybridized with TRITC-labelled probe LEG 705.

In situ detection of *L. pneumophila* in model biofilms. A model biofilm of a water bacterium identified as *Sphingomonas paucimobilis* by API 20 NE (Analytab Products) was generated using a down-flow fixed-bed reactor as described recently (Szewzyk *et al.*, 1994). After 2 d, established biofilms were transferred to a continuous flow unit and inoculated with low numbers (10^2 – 10^3 c.f.u. ml⁻¹) of *L. pneumophila* serogroup 1 strain 56/1 (SMI culture collection). One hour after inoculation continuous flow was started to remove unattached *L. pneumophila* cells. The glass slides were removed after 8, 12 and 24 h incubation and subsequently used for *in situ* hybridization (Manz *et al.*, 1993).

In situ hybridization of *L. pneumophila* within fixed cells of *Tetrahymena pyriformis*. *T. pyriformis* was grown on synthetic freshwater medium (Szewzyk *et al.*, 1994) at 32 °C supplemented with heat-killed *Escherichia coli* as food bacteria. Cells of a water isolate of *L. pneumophila* serogroup 1 (strain SJ 56/1, SMI culture collection) were added at concentrations of 10^8 or 10^9 cells ml⁻¹, respectively. After 3 d incubation (37 °C) cells of *T. pyriformis* were transferred onto sterile glass slides and air-dried. Finally, slides containing cells of *T. pyriformis* were fixed for 1 h in 4% (w/v) aqueous paraformaldehyde solution, washed once in PBS and dehydrated in an aqueous ethanol series (50%, 80%, 96%, v/v). For *in situ* hybridization aliquots of 20 µl hybridization buffer (20%, v/v, formamide, 0.9 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.6) containing 100 ng of the labelled probe were dotted onto the slides followed by hybridization for 1.5 h at 46 °C in a moisture chamber. The probe was removed gently with washing buffer (20 mM Tris/HCl, pH 7.6, 180 mM NaCl, 0.01% SDS), and the cells

Table 2. *Legionella* strains used for whole-cell and dot-blot hybridizations

Name	Origin*	Reaction with probe†	
		LEG226	LEG705
<i>Legionella pneumophila</i> sg 1	ATCC 33152 ^T	+	+
<i>Legionella pneumophila</i> sg 2	ATCC 33154	+	+
<i>Legionella pneumophila</i> sg 3	ATCC 33155	+	+
<i>Legionella pneumophila</i> sg 4	ATCC 33156	+	+
<i>Legionella pneumophila</i> sg 5	ATCC 33216	+	+
<i>Legionella pneumophila</i> sg 6	ATCC 33215	+	+
<i>Legionella pneumophila</i> sg 7	ATCC 33823	+	+
<i>Legionella pneumophila</i> sg 8	ATCC 35096	+	+
<i>Legionella pneumophila</i> sg 9	ATCC 35289	+	+
<i>Legionella pneumophila</i> sg 10	ATCC 35283	+	+
<i>Legionella pneumophila</i> sg 11	ATCC 43130	+	+
<i>Legionella pneumophila</i> sg 12	ATCC 43290	+	+
<i>Legionella pneumophila</i> sg 13	ATCC 43736	+	+
<i>Legionella pneumophila</i> sg 14	ATCC 43703	+	+
<i>Legionella anisa</i>	ATCC 35292 ^T	+	+
<i>Legionella bozemanii</i> sg 1	ATCC 33217 ^T	+	+
<i>Legionella bozemanii</i> sg 2	ATCC 35545	+	+
<i>Legionella cicinnatiensis</i>	ATCC 43753 ^T	+	+
<i>Legionella dumoffii</i>	ATCC 33279 ^T	+	+
<i>Legionella erythra</i>	ATCC 35303 ^T	+	+
<i>Legionella feeleeii</i>	ATCC 35072 ^T	+	+
<i>Legionella gormanii</i>	ATCC 33297 ^T	+	+
<i>Legionella hackeliae</i>	ATCC 35250 ^T	+	+
<i>Legionella hackeliae</i>	ATCC 35999	+	+
<i>Legionella jordanis</i>	ATCC 33623 ^T	+	+
<i>Legionella longbeachae</i> sg 1	ATCC 33462 ^T	+	+
<i>Legionella micdadei</i> Tatlock	ATCC 33218 ^T	+	+
<i>Legionella rubrilucens</i>	ATCC 35304 ^T	+	+
<i>Legionella saintelensis</i>	ATCC 35248 ^T	+	+
<i>Legionella spiritensis</i>	ATCC 35249 ^T	+	+
<i>Legionella tucsonensis</i>	ATCC 49180 ^T	+	+
<i>Legionella wadsworthii</i>	ATCC 33877 ^T	+	+
<i>Legionella birminghamensis</i>	ATCC 43702 ^T	—	+
<i>Legionella feeleeii</i>	ATCC 35849	—	+
<i>Legionella oakridgensis</i>	ATCC 33761 ^T	—	+
<i>Legionella israelensis</i>	ATCC 43119 ^T	—	+
<i>Legionella parisiensis</i>	ATCC 35299 ^T	—	—
<i>Legionella quinlivanii</i>	ATCC 43830 ^T	—	—

* T, Type strain.

† +, Strong hybridization signal; —, no hybridization signal.

were overlaid with 500 µl washing buffer and incubated in a moisture chamber for 15 min at 46 °C. Finally, the slides were carefully rinsed with distilled water, air-dried, and mounted in Citifluor.

Microscopy and documentation. Probe-conferred fluorescence was detected with Zeiss Axioplan and Zeiss Axioskop microscopes fitted for epifluorescence microscopy with 50 W high-pressure bulbs and Zeiss filter sets 01, 09 and 15. Colour-

micrographs were taken with Fuji P 1600 and Kodak EES 1600 colour reversal film. Exposure times were 2–8 s for epifluorescence micrographs and 0.01–0.03 s for phase contrast. Alternatively, confocal epifluorescence laser scanning microscopy was done on a Carl Zeiss LSM 410 using an excitation wavelength of 543 nm. A series of 24 confocal sections were scanned with increments of 0.5 µm. Image processing and three-dimensional reconstructions were performed using the standard software package delivered with the instrument.

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

Probe	Sequence	Target site*	Formamide (%)
LEG226	5'-TCGGACGCAGGCTAATCT-3'	16S, 226–243	20
LEG705	5'-CTGGTGTTCTTCCGATC-3'	16S, 705–722	20
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	16S, 338–355	0
Alf1b	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16S, 19–35	20

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

Fig. 1. *In situ* hybridization of infected *T. pyriformis* cells with TRITC-labelled probe LEG705. Phase-contrast (left) and epifluorescence micrographs (right) are shown for identical microscopic fields. All photomicrographs were done at a magnification of $\times 1000$. (a) Cells of *L. pneumophila* are arranged within vacuoles of *T. pyriformis*; (b) cells of *L. pneumophila* being released from a former vacuole in the process of colonizing the protoplasm.

RESULTS

Probe specificities

The probes specific for the family *Legionellaceae* (Table 3) were designed based on comparative analysis of about 30 complete or almost complete 16S rRNA-sequences of *Legionellaceae* (Fry *et al.*, 1991b) and furthermore approximately 1500 sequences of other bacteria (Larsen *et al.*, 1993; Neef *et al.*, 1993). With the exception of the 16S rRNA sequence of *Methylomonas alba*, which is complementary to probe LEG705, the probes LEG226 and LEG705 were complementary only to the target regions on the 16S rRNA sequences of members of the *Legionellaceae* and the legionella-related intracellular parasite *Sarcobium lyticum* (Springer *et al.*, 1992). All of the other available 16S rRNA sequences in the databases showed at least one mismatch. As we could demonstrate previously (Amann *et al.*, 1990a; Manz *et al.*, 1992) oligonucleotide probes can distinguish between complementary and nearly complementary sequences on the basis of a single mismatch.

Since 16S rRNA sequences have only been determined for a fraction of the described bacterial species probe specificities were further evaluated by dot-blot analysis against 72 non-legionellae reference strains (Table 1) and 22 different species of *Legionella* (Table 2). The optimal hybridization stringencies were determined by gradually increasing (in 5% intervals) the formamide concentration in the hybridization buffer while holding ionic strength (0.9 M NaCl) and hybridization temperature (46 °C) constant. At 20% (v/v) formamide probes LEG705 and LEG226 hybridized to all tested strains of *L. pneumophila* and most of the *Legionella* spp. Whereas probe LEG705 hybridized to all legionellae except for *L. parisiensis* and *L. quinlivanii* (Table 2), probe LEG226 has a somewhat narrower specificity and additionally failed to detect *L. israelensis*, *L. oakridgensis*, *L. birminghamensis* and one of two tested strains of *L. feeleii*. No false-positive hybridizations with the reference strains (Table 1) occurred. The immobilization of nucleic acids to the membranes was checked by hybridization with the positive control EUB338 (Amann *et al.*, 1990a); there were positive hybridization signals with all immobilized nucleic acids.

Whole-cell hybridization with probe LEG705 allowed the differentiation of *Legionella* spp. from reference strains like *E. coli* even at low stringency conditions (i.e. without addition of formamide). Nevertheless, to assure the specificities determined with the dot blots, whole-cell hybridizations were performed at a formamide concentration of 20%. Whereas good signal strength and specificity was found for LEG705 under these conditions,

probe LEG226 yielded weaker fluorescent signals. Therefore, probe LEG705 was used for all whole-cell hybridizations.

Rapid determination of environmental and clinical isolates by whole-cell hybridization

To evaluate the usefulness and practicability of the whole-cell hybridization assay, 130 isolates obtained on BCYE agar inoculated with environmental and clinical samples were examined. Within a collection of 59 isolates which were considered to be members of *Legionellaceae* by an immunofluorescence assay, 58 isolates showed strong hybridization signals when hybridized with probe LEG705. Further testing of the non-reacting isolate revealed physiological properties uncommon to legionellae. Testing of the 59 isolates by *in situ* whole-cell hybridizations could be performed within 1 working day.

Whole-cell hybridization on membrane filters

Membrane filtration is a standard technique to quantify microbial cells in water samples. Cells of *L. pneumophila* collected from tap water samples seeded with approximately 10^3 – 10^4 c.f.u. ml⁻¹ were readily visualized on the polycarbonate filters by hybridization with the TRITC-labelled probe LEG705. When filters were mounted in immersion oil or distilled water probe-conferred fluorescence faded very rapidly, whereas mounting in Citifluor strongly decreased photobleaching and allowed microscopy evaluation even after 48 h.

Whole-cell hybridization of starved cells of *L. pneumophila*

Cells of *L. pneumophila* strain 56/1 could not be cultivated on BCYE agar after 1 year of incubation in sterile drinking water at 30 °C. However, hybridization with probe LEG705 demonstrated the presence of *Legionella* cells. Approximately one third of the cells detected by phase contrast microscopy showed detectable levels of probe-conferred fluorescence after hybridization with probe LEG705.

In situ identification of *L. pneumophila* in model biofilms

One important advantage of fluorescently labelled oligonucleotide probes is their potential for *in situ* visualization of the respective target populations. They have already successfully been used to detect sulfate-reducing bacteria

Fig. 2. Confocal fluorescence micrographs of infected *T. pyriformis* cells hybridized with TRITC-labelled probe LEG705. Twenty-four confocal sections were scanned along the optical axis with increments of 0.5 µm. (a) Collection of four typical optical sections taken at depths of 1 µm, 3.5 µm, 6 µm and 8.5 µm showing individual cells of *L. pneumophila* within the host cell. (b) Transparent projection of all 24 confocal sections scanned in distances of 0.5 µm resulting in an extended focus image of the *Legionella* cell cluster within *T. pyriformis*. (c) Interpolated projection perpendicular to image (b) showing the three-dimensional intracellular arrangement of *L. pneumophila* cells within *T. pyriformis*.

in complex multispecies biofilms (Amann *et al.*, 1990b; Ramsing *et al.*, 1994) and for the detection of *E. coli* in a model biofilm formed by an aquatic isolate (Szewzyk *et al.*, 1994). In a similar experiment biofilms formed by *S. paucimobilis* were examined for attachment and growth of *L. pneumophila* after 8, 12 and 24 h of incubation. As expected, probe LEG705 facilitated *in situ* monitoring of *L. pneumophila* (data not shown). Legionellae did attach to the model biofilm and could be detected after 12 h; in this time the volume of the continuous flow unit had been exchanged 40 times. Further studies will examine the potential of legionellae to survive and multiply in such biofilms.

In situ hybridization of infected cells of *T. pyriformis*

It has been reported that *Legionellaceae* are able to infect and proliferate within various types of protozoa (Fry *et al.*, 1991a) including *T. pyriformis* (Fields *et al.*, 1984). *In situ* hybridization of infected *Tetrahymena* cells with probe LEG705 visualized by epifluorescence microscopy corroborated these findings. With 10^8 c.f.u. ml⁻¹ of *L. pneumophila* as inoculum, the cells were tightly packed in certain areas, probably phagosomes, within *T. pyriformis* (Fig. 1a). However, some cells of *L. pneumophila* appeared to be spread over the host cell in the experiments with 10^6 c.f.u. ml⁻¹ as inoculum (Fig. 1b). Additionally, *L. pneumophila* cells with bright fluorescence were observed in free phagosomes outside the protozoan cells. Using CLSM, the limitations of conventional epifluorescence microscopy including out-of-focus haze, could be overcome. Optical sections of the specimen enabled determination of the exact three-dimensional localization of the *L. pneumophila* cells inside the ciliate. This demonstrated that there were indeed two separate stages of infection, depending on the initial cell concentration. At higher initial cell concentrations, the *Legionella* remained restricted to small spherical areas, whereas at lower initial cell concentrations they filled nearly the whole lumen of the ciliates. The three-dimensional structure of the *L. pneumophila* cell-cluster within the host cell is elucidated by a collection of four typical optical sections taken at depths of 1 µm, 3.5 µm, 6 µm and 8.5 µm (Fig. 2a). The resulting transparent projection of all optical sections in an extended focus image (Fig. 2b) is supplemented by the perpendicular projection (Fig. 2c), which is interpolated from the sections along the optical axis with increments of 0.5 µm.

DISCUSSION

The family *Legionellaceae* includes members which act as pathogens in both humans and protozoa as well as members so far only described as pathogens of amoebae (Rowbotham, 1980, 1993). It is assumed that various free-living protozoa are the natural hosts for most members of the *Legionellaceae* and that intracellular growth is a prerequisite or at least an advantage for life in natural water environments (Fields, 1993). *Legionella* spp. have been shown to preferentially live in biofilms where close interaction with other water micro-organisms is possible

(Henke & Seidel, 1986; Wadowsky *et al.*, 1988; Fields *et al.*, 1989). However, most of the discussion dealing with the ecology of *Legionella* spp. is speculative and needs further verification. One tool that could help to further examine the behaviour of *Legionella* spp. in complex microbial communities and diverse ecosystems is the application of rRNA-targeted oligonucleotide probes, which combine defined specificity with an indication of the physiological state of the detected cell.

The probes developed during this project were specific for most members of the *Legionellaceae*. In particular, clinically relevant species of the family *Legionellaceae*, such as *L. pneumophila*, *L. longbeachae*, *L. micdadei* and *L. bozemanii*, tested positive with our probes. Since isolates of *Legionellaceae* from environmental samples are characterized for the most part as *L. pneumophila* (Bartlett *et al.*, 1983; Habicht & Müller, 1988) the probes are also of great value for ecological studies.

Probes LEG705 and LEG226 also hybridized to *S. lyticum*, an obligate intracellular parasite closely related to the *Legionellaceae* (Springer *et al.*, 1992; Rowbotham, 1993). A 16S rRNA database comparison revealed that the target sequence of probe LEG705 is also present in *M. alba*, a methane-oxidizing bacterium. These bacteria occur where both methane and oxygen are present, for example in the water and mud above anaerobic sediments (Hansen, 1980). The value of probe LEG705 for analysis of clinical and drinking water samples is therefore not compromised. As for the specificity of probe LEG226, within the available database which comprises more than 1500 complete or almost complete 16S rRNA sequences only members of the family *Legionellaceae* showed complementarity to the probe sequence. On the other hand, probe LEG226 revealed a somewhat narrower specificity and weaker fluorescent signals during *in situ* whole-cell hybridizations than probe LEG705.

Specific detection of *Legionella* spp. in natural environments has so far mainly been done by using antibodies or PCR and related techniques (Fliermans *et al.*, 1981; Makin & Hart, 1989; Rogers & Keevil, 1992; Koide *et al.*, 1993; Palmer *et al.*, 1993). Whole-cell probing with rRNA-targeted oligonucleotides will be an important addition to these established techniques. The use of antibodies in natural samples is often hampered by non-specific binding to other bacteria or even detritus particles. Furthermore, biofilms have been shown to act as penetration barriers for antibodies making detection of cells in deeper layers of biofilms a difficult task (Szwedinski *et al.*, 1985).

In this study, 16S rRNA-targeted probes were successfully used to detect *Legionella* spp. in both natural water samples and model biofilms without non-specific fluorescence. Detection of *Legionellaceae* with the 16S rRNA probes was also possible on membrane filters without interference from background fluorescence. This combination of techniques could facilitate the rapid detection of *Legionella* spp. in water samples. It should, however, be considered that for any microscopic method, around 10^4 cells have to be collected on a 1-inch filter in order to detect one cell per microscopic field. Therefore, PCR or culture techniques

may be more efficient for detecting low levels of contamination.

The value of culture techniques is compromised by the occurrence of viable but non-culturable cells of *Legionella* spp. which have been demonstrated for various natural, experimental, and industrial systems (Colbourne *et al.*, 1988; Bej *et al.*, 1991; Paszko-Kolva *et al.*, 1992; Miller *et al.*, 1993). Here, oligonucleotide probes were used for the detection of non-culturable *L. pneumophila* cells (as assessed on BCYE agar) in sterilized drinking water after 1 year of incubation. Approximately one third of the cells showed detectable fluorescence signals with the 16S rRNA probes. These cells were assumed to be viable but non-culturable whereas the others may be non-viable cells. These results indicated that like PCR or other gene probe techniques (Bej *et al.*, 1991; Paszko-Kolva *et al.*, 1992; Miller *et al.*, 1993) rRNA-targeted oligonucleotide probing can be used to detect non-culturable *Legionellaceae*. The great advantage of the 16S rRNA probes over PCR is that they are not only specific for detection of bacteria but they also give information on the metabolic activity and the spatial distribution of the detected cells. The detection of *L. pneumophila* was possible inside of eukaryotic cells or biofilms by use of fluorescently labelled rRNA probes. Comparable *in situ* detection is difficult to achieve with antibodies due to the diffusion and penetration barriers.

The application of CLSM allowed the reconstruction of the spatial arrangement of the *Legionellaceae* inside the protozoan host cells. Similar, yet less detailed observations on the invasion of amoebae and ciliated protozoa by *Legionella* spp. and the intracellular growth of legionellae have been made previously with conventional techniques (Rowbotham, 1980; Fields, 1993). It has been shown that active phagocytosis is necessary for the uptake of *Legionella* spp. into amoebae, and single cells of *Legionella* spp. could be found immediately after entry within a special kind of phagosome, the so-called endosome (Fields, 1993). In this study *Legionella* spp. could be identified both in defined subcellular compartments and randomly in the cytoplasm. The strong fluorescent signals of the intracellular legionellae reflected a high cellular ribosome content and therefore a high physiological activity. High metabolic activity of the intracellular *Legionella* spp. has also been shown by Rowbotham (1986), based on the observation of highly motile intracellular *Legionella* spp. cells in certain stages of infection. Hybridization with the 16S rRNA probes also revealed that densely packed aggregates of highly active *L. pneumophila* cells developed in the protozoa and were released by lysis of host cells.

Fluorescent oligonucleotide probes like LEG705 combined with CLSM will in the future help to monitor the spatial and temporal distribution of microbial populations with a resolution that is not possible with conventional techniques. The consequent use of the 16S rRNA probes will help to further examine and understand the ecology of micro-organisms with complex growth requirements, such as the members of the *Legionellaceae*. It should be also

feasible to elucidate whether, as frequently reported, sudden high contamination of water by *Legionella* spp. is indeed due to proliferation in protozoa and how other processes like survival and multiplication in biofilms may also contribute to this phenomenon. Furthermore, these probes are quick and easy tools for the detection and identification of *Legionella* spp. in routine diagnosis.

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