System. Appl. Microbiol. 20, 310–318 (1997)
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Development and Use of Fluorescent *In Situ* Hybridization Probes for the Detection and Identification of *"Microthrix parvicella"* in Activated Sludge

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Received December 10, 1996

Summary

Four 16S rRNA directed oligonucleotide probes (MPA probes) specific for the activated sludge bulking and foaming filamentous bacterium *"Microthrix parvicella"* were designed and evaluated for the *in situ* detection and identification of this organism. A method for successful permeabilization of *"M. parvicella"* cells employing mutanolysin was developed. Hybridization stringency for the probes was empirically determined with activated sludge samples because *"M. parvicella"* cannot be cultured to give adequate amounts of biomass. A probe complimentary to the 16S rRNA of most bacteria (EUB338) was used to confirm the presence and accessibility of sufficient numbers of ribosomes in *"M. parvicella"*. None of a wide range of pure cultures of bacteria gave positive hybridization signals with any of the MPA probes. Three of the developed probes (MPA60, MPA223 and MPA645) were highly specific for filaments morphologically identified as *"M. parvicella"* in activated sludge samples while one probe (MPA650), required the use of two competitor probes to be highly *"M. parvicella"*-specific. None of the cells morphologically identified as *"M. parvicella"* gave positive hybridization signals with a previously reported probe for high mol%G+C gram positive bacteria. *"M. parvicella"* filaments in activated sludge plants from Australia, France and Germany bound all four MPA probes suggesting that the same genotype is present in each of these countries. A combination of *in situ* hybridization probing and staining with DAPI showed segments of the *"M. parvicella"* filaments that contained large amounts of polyphosphates were low in ribosomes. From this, we concluded that the storage of polyphosphates could be a survival strategy for *"M. parvicella"*.

Key words: *"Microthrix parvicella"*– activated sludge – bulking – foaming – *in situ* hybridization – oligonucleotide probes – 16S rRNA

Introduction

"Microthrix parvicella" is a straight-curved, filamentous, gram positive bacterium that causes bulking and foaming in activated sludge sewage treatment plants throughout the world (BLACKBEARD et al., 1988; JENKINS et al., 1993; PUJOL et al., 1991; ROSSETTI et al., 1994; SEVIOUR et al., 1990). PASVEER (1969) first described *"M. parvicella"* from a Dutch bulking sludge plant and shortly after, others reported the presence of this characteristic and somewhat irregular and winding filament (EIKELBOOM, 1975; FARQUHAR and BOYLE, 1971a; VAN VEEN, 1973). FARQUHAR and BOYLE (1971a, 1971b) hypothesised that *"M. parvicella"* could be a filament-forming lactic-acid bacterium and it has now been shown that *"M. parvicella"* is a deep branching member of the actinomycetes subphylum (BLACKALL et al., 1994). Some of the currently known closest relatives of *"M. parvicella"* are members of the genus *Atopobium*, a bacterium which was originally thought to belong in the low mol%G+C gram positive subphylum along with the lactic acid bacteria (STACKEBRANDT and LUDWIG, 1994).

VAN VEEN (1973) gave *"M. parvicella"* its name and carefully described a number of its cardinal attributes including cellular morphology, trichome dimensions and staining properties, and presence of intracellular inclusions. The extremely slow growth rate of *"M. parvicella"*, the difficulty in storing the isolates, and the inability to generate phenotypic information was noted by many of the early workers, including FARQUHAR and BOYLE (1971a), and VAN VEEN (1973).

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The phylogenetic position of *"M. parvicella"* in the high mol%G+C gram positive subphylum (the actinomycetes) was determined by 16S rRNA comparative analysis (BLACKALL et al., 1994), and we have employed the *"M. parvicella"* 16S rRNA sequence to prepare specific rRNA directed, DNA-hybridization probes for the *in situ* identification of this bacterium. This paper reports the design and use of these *"M. parvicella"* specific whole-cell probes. In addition, we report the evaluation of the high mol% G+C gram positive probe for its ability to bind to *"M. parvicella"* filaments in activated sludge samples. This probe was reported to bind to *"M. parvicella"* filaments *in situ* following diethyl ether pretreatment but under these conditions, the gram negative bacteria in samples produced reduced hybridization signals (WAGNER et al., 1994).

Materials and Methods

Sampling: Grab samples of mixed liquor from three full scale activated sludge plants in Germany (Willstaedt and Hirblingen) and France (Troyes) and three in Australia (Bendigo, Dandenong, and Orange) were collected and were immediately fixed with a 1:1 ratio with 100% ethanol or with paraformaldehyde/ethanol (WAGNER et al., 1994). The samples were then stored at –20 °C. Additional unfixed samples were collected at the same time for the microscopic morphological identifications of the filamentous bacteria present in the plants (EIKELBOOM and VAN BUIJSEN, 1981). Some technical data relevant to these activated sludge plants are given in Table 1.

In situ probing: Total cells were determined by a combination of membrane filtration and staining with the DNA-specific fluorescent dye DAPI (PORTER and FEIG, 1980). The presence of *"M. parvicella"* in activated sludge samples was determined with fluorescently-labeled, rRNA-directed oligonucleotide probes using methods described earlier (WAGNER et al., 1993). The EUB338 probe which binds to all *Bacteria* (AMANN et al., 1990), the high mol%G+C gram positive (HGC69a) probe designed by ROLLER et al. (1994) and four specifically-designed probes for *"M. parvicella"* (MPA probes) were used (Table 2). Two competitor oligonucleotides for MPA650 were also employed (Table 2). Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoracetylamino)-hexyl-(2-cyanoethyl)-(*N,N*-diisopropyl) phosphoramidite] at the 5′end (MWG Biotech, Ebersberg, Germany). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC, Molecular Probes, Eugene, Oregon), 5(6)-carboxytetramethylrhodamine-succinimidyl-ester (CT, Molecular Probes), 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS, Boehringer GmbH, Mannheim, Germany), and the indocarbocyanine dye CY3 (Biological Detection Systems, Inc, Pittsburgh, USA), and purification of the oligonucleotide-dye conjugates were performed as described by AMANN et al. (1990). Some fluorescein-labelled oligonucleotides were obtained from Bresatec (Bresatec, Adelaidem Australia) and some rhodamine-labelled oligonucleotides were purchased from Eurogentec (Eurogentec, Belgium).

Cell fixation and permeabilization: Approximately 3 µl of fixed samples were applied to gelatin-coated slides (MANZ et al., 1992). The samples on slides were then pretreated to permeabilize the cells allowing the *in situ* hybridization probes to enter the cells. A range of methods were evaluated including acid hydrolysis reported by MACNAUGHTON et al. (1994); diethylether for times varying between 1 and 30 min; 1,4 dithio-L-threitol at 0.025 M, 0.05 M and 0.125 M with and without 1% SDS and for 30 sec, 1 min, 2 min, 5 min, or 10 min (FISCHER et al., 1995); mutanolysin (Fluka, 5000 U/ml in 0.1 M potassium phosphate buffer at pH 6.8); and lysozyme (from chicken egg white, Serva, 150,000 U/mg at 10 mg/ml and 1 mg/ml in 100 mM TRIS, 50 mM EDTA, pH 8.0). Ethanol treatments (50%, 80% and 98% for 3 min each) were carried out before and after the enzyme treatments.

Hybridization and microscopy: Apart from the above permeabilization treatments, hybridizations of fixed, activated sludge samples on microscope slides were carried out in a hybridization buffer containing 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% SDS and varying amounts of formamide to achieve values from 0% through to 60%. Hybridization was performed for 1.5 h at 46 °C in an isotonically equilibrated humid chamber and followed by a 20 min washing step at 48 °C. In order to achieve the same stringency during washing as during hybridization, the washing buffer contained between 0.9 M and 7 mM NaCl according to the formula of LATHE (1985) applied with a destabilization increment for the DNA:RNA hybrids of 0.5 °C per % formamide, 20 mM Tris/HCl (pH 7.4) and 0.01% SDS. For combinations of probes with different optimal hybridization stringencies, two hybridizations with the appropriate formamide concentrations were done successively. The first hybridization was performed with the probes(s) which required the higher formamide con-

Table 1. Technical data of activated sludge plants in which *"Microthrix parvicella"* was detected.

Plant[a]	Aerators	Wastewater	Sludge age (d)	SV30[b] (g/ml)	MLSS[c] (g/l)
1. Dandenong	mechanical	domestic/industrial	19	950	2.2
2. Bendigo	mechanical	domestic	25	150–200	4.2
3. Orange	mechanical	mostly domestic	20	206	3.9
4. Willstaedt	small bubble, ceramic diffusers	domestic (60)/ industrial (40)	14	500–600	3.5–5.6
5. Hirblingen	diffusers	domestic	10–12	500–550	6.0–7.5
6. Troyes	bottom diffusers and propellers	domestic/industrial	20	160	4.15

[a] Plants 1–3 are from Australia, plants 4 and 5 from Germany, and plant 6 from France;
[b] Sludge volume index at 30 min;
[c] Mixed liquor suspended solids.

Table 2. *"Microthrix parvicella"* probe sequences and target sites for the *in situ* hybridization.

Probe	Sequence (5´->3´)	Target site (16S rRNA position)[a]
MPA60	GGATGGCCGCGTTCGACT	60–77
MPA223	GCCGCGAGACCCTCCTAG	223–240
MPA645	CCGGACTCTAGTCAGAGC	645–661
MPA650	CCCTACCGGACTCTAGTC	650–666
CompMPA650.1	CCCTACCGCACTCTAGTC#	650–666
CompMPA650.2	CCCTACCGAACTCTAGCC#	650–666

[a] From *Escherichia coli* numbering of BROSIUS et al. (1981);
Underlined nucleotides indicate the variation from MPA650.

centration and followed by a second hybridization at the lower stringency with the other probe(s).

Optimal hybridization stringency required the addition of formamide to a final concentration of 20% (for EUB338) and 25% (for HGC69a) in the hybridization buffer. Optimization of hybridization conditions for the newly developed probes on isolated rRNA or cultured cells of *"M. parvicella"* was not possible since we were unable to obtain adequate amounts of *"M. parvicella"* biomass. Therefore we tested each probe at various formamide concentrations between 0 and 60% using activated sludge samples containing morphologically identified *"M. parvicella"*. Each probe except MPA645 (40%) was stable up to 50% formamide. All the results with MPA probes reported in this paper employed 20% formamide in the hybridization buffer.

The probe specificities were also evaluated by simultaneous hybridization of differently labelled MPA probes.

Hybridized samples were mounted in antibleaching buffer (Citifluor, Citifluor Ltd., Canterbury, UK). Slides were routinely examined with either an Axioplan microscope (Zeiss, Oberkochen, Germany) with filter sets 09 and 15 or with a Nikon Microphot-FXA/SA microscope (Nikon, Sydney, Australia) with filter blocks V-2B, B-2A and G-2A. Some samples were also examined using a Bio-Rad MRC 600 confocal scanning laser microscope mounted on a Zeiss Axioskop equipped with a Zeiss Plan-APOCHROMAT 63X oil immersion objective.

Cells of *Gordona amarae* were used as positive controls for the EUB338 and HGC69a probes. A range of pure cultures of gram positive bacteria (ROLLER et al., 1994; SCHUPPLER et al., 1995) were evaluated with the MPA probes with varying formamide concentrations from 0% to 60% (5% increments) in the hybridization buffer. The specificity of probe MPA650 and of the competitor probes (CompMPA650.1 and CompMPA 650.2) was performed with equimolar amounts of unlabelled competitor and probe according to MANZ et al. (1992). The evaluation was done with 120 high mol% G+C gram positives and eight low mol% G+C gram positives to check possible cross reactions of the MPA probes with 23S rRNA and 16S rRNA. In addition, a search for other MPA probe targets in the 16S rRNA was done in an aligned dataset of over 6,000 complete and partial 16S rRNA sequences (LUDWIG, 1995; MAIDAK et al., 1994) using the ARB software (STRUNK et al., in press).

Intracellular inclusions and cell viability: Fresh ethanol-fixed samples, with and without mutanolysin treatment, were hybridized with the whole-cell probes and stained with DAPI (1 µg/ml, WAGNER et al., 1994). The slides were examined by epifluorescene microscopy. They were then stained for polyphosphate and poly-β-hydroxyalkanoates according to methods in REES et al. (1992) and additionally for polyphosphate by the Neisser method as described by EIKELBOOM and VAN BUIJSEN (1981).

The effect of sample storage conditions on stainability was also evaluated. Ethanol- and paraformaldehyde/ethanol-fixed samples which had been stored for 2 years were hybridized with the whole-cell probes and DAPI stained. Staining for poly-β-hydroxyalkanoates and polyphosphate (EIKELBOOM and VAN BUIJSEN, 1981; REES et al., 1992) was also carried out on both fresh and stored samples.

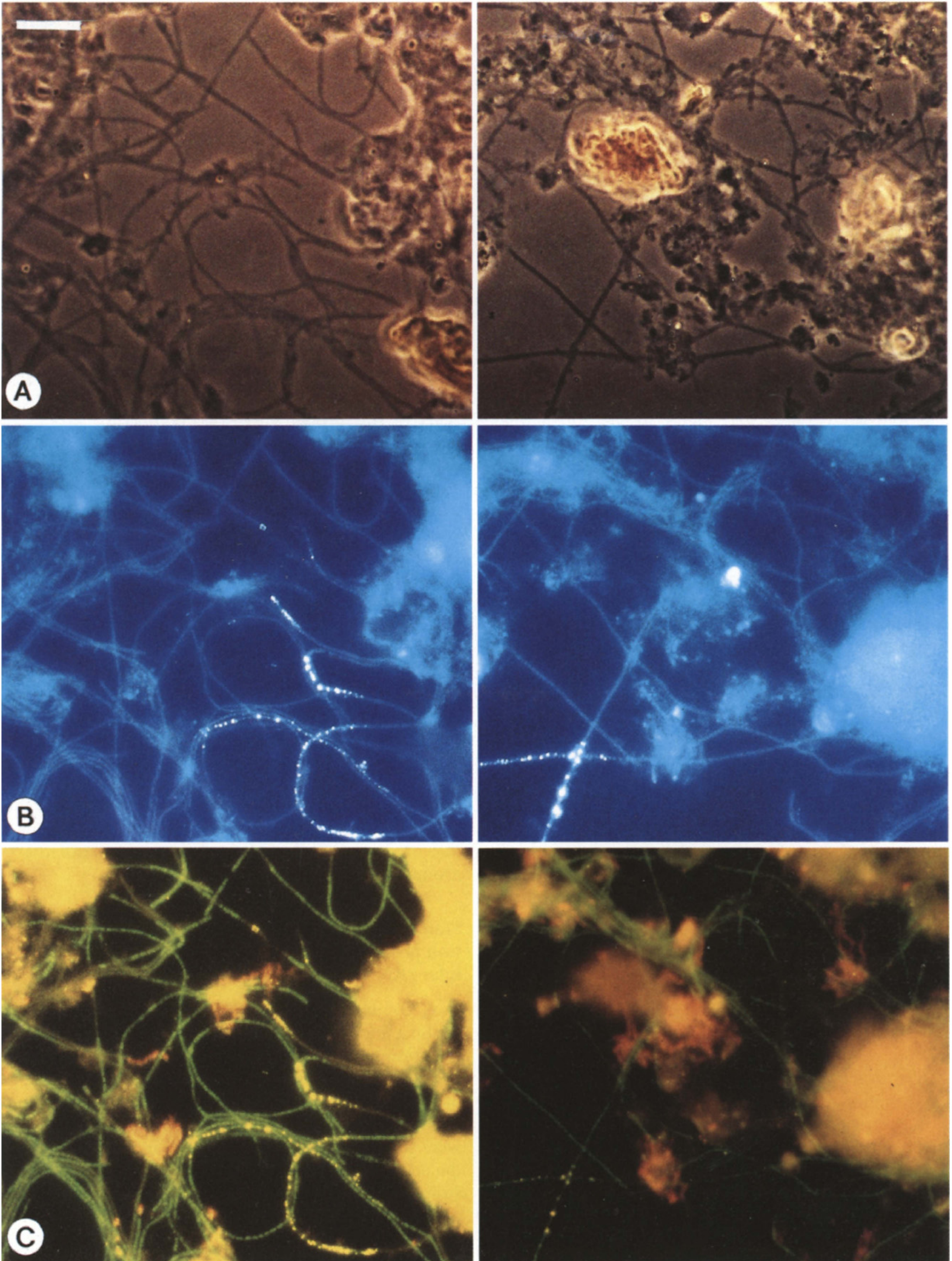
Results

"M. parvicella" filaments were identified in mixed liquor samples according to the keys in EIKELBOOM and VAN BUIJSEN (1981). In addition, filaments of *"M. parvicella"* were clearly visualized by DAPI staining, indicating that the cells contained nucleic acids (Fig. 1 B).

Permeabilization

Permeabilization methods described by MACNAUGHTON et al. (1994), and the employment of diethyl ether and 1,4 dithio-L-threitol with and without SDS (FISCHER et al. 1995) as described in the Materials and Methods section did not facilitate visualization of cells morphologically identified as *"M. parvicella"* when samples were probed with the EUB338 probe. Both mutanolysin and lysozyme facilitated the entry of the EUB338 probe into *"M. parvicella"* filaments. Mutanolysin treatment resulted in a more even staining of the *"M. parvicella"* filaments when compared to lysozyme treatment. For optimal results, enzyme treatment times varied depending upon sample age. A two year old fixed sample from Hirblingen, Germany, required 10 min exposure to mutanolysin, while fresh samples required 20 min exposure to achieve even staining. More intense fluorescence of *"M. parvicella"* filaments was obtained with the fresh samples after a 35 min exposure to mutanolysin. In these experiments, comparisons between different mutanolysin exposures were made with constant hybridization times and probe concentrations.

Fig. 1. *In situ* identification of *"Microthrix parvicella"* in activated sludge samples.
Left column: Activated sludge sample obtained from the wastewater treatment plant Hirblingen, Germany.
Right column: Activated sludge sample obtained from the wastewater treatment plant Willsteadt, Germany.
For each column, identical fields were viewed by phase contrast microscopy (panel **A**) and epifluorescence microscopy (panels **B** and **C**).
DAPI staining reveals yellowish-white fluorescent polyphosphate granules within the *"Microthrix parvicella"* filaments (panel **B**). Hybridization with probe HGC69a-CT and MPA650-FLUOS (double exposure, panel **C**) shows different populations. The DAPI-stained fluorescent polyphosphate granules are still visible within the filaments in yellow. Background fluorescence in panel C can be seen as indistinct, yellow material.
Bar (panel **A**, left) represents 10 µm and applies to all micrographs.


A
B
C

"M. *parvicella*" filaments in the sludge samples showed strong fluorescence when probed with EUB338 indicating that they are synthesising sufficient ribosomes to be detected by *in situ* hybridization and that they have a high potential for metabolic activity.

Probing for "M. *parvicella*"

All results with the MPA probes employed mutanolysin treatment for 15–25 min and to accommodate the use of the MPA probes in combination with either EUB338 or HGC69a probes, 20% formamide was used in the hybridization buffer. The determination of melting profiles for the probes was desirable but by being restricted to sludge material for evaluation, this determination was strongly hampered by natural autofluorescence of the activated sludge flocs (see the yellow material in Fig 1C).

The four MPA probes were highly specific for the filaments identified as "M. *parvicella*" (Fig 1). In samples lacking morphologically-identified "M. *parvicella*" filaments, no bacterial cells bound all four MPA probes. However, MPA650 bound to cells not identified as "M. *parvicella*" and so competitor probes were prepared and evaluated (see below). "M. *parvicella*" filaments were shown to be non-branching, approximately 0.5 μm in diameter, and although filaments 200–400 μm long were seen, shorter filaments were also present. However, the cell septa within the filaments were occasionally observed. Cell septa are typically not observed in filaments of "M. *parvicella*" and our ability to observe septa may be as a result of the methods used for sample fixation and cell permeabilization.

Simultaneous hybridizations were also done and, for example when a FLUOS labelled probe MPA223 was used in combination with a CT labelled probe MPA645, all cells visualized as green were also visualized as red thus indicating matching target populations. All six possible combinations of the four MPA probes were evaluated and always resulted in dual staining of morphologically identified "M. *parvicella*" filaments (data not shown). None of the filaments detectable with all MPA probes could be detected with probe HGC69a or with any other group specific probe so far available such as those for the cytophaga-flavobacter-bacteroides phylum (MANZ et al., 1996) or the subclasses of the proteobacteria (MANZ et al., 1992).

None of the cells morphologically identified as "M. *parvicella*" gave positive hybridization signals with the HGC69a probe. However, other cells in the activated sludge samples and pure cultures of *G. amarae* showed high fluorescence when probed with the probe HGC69a indicating that this probe was working efficiently. None of the pure culture tested bound to the MPA probes, an observation which agrees with sequence database information.

Table 3 shows the different alignments of target sequences, matching target organisms and examples of non-target organisms for the designed MPA probes. All matching species as well as all species with one mismatch and examples of species with greater than one mismatch for the MPA probes are shown. An incomplete list of target species for the competitor probes is also shown. Additionally, Table 4 shows the reference strains of choice according to the database search.

Survey of plants for "M. *parvicella*"

Filaments morphologically identified as "M. *parvicella*" in samples from activated sludge plants in Germany, France and Australia bound to all four MPA probes suggesting that the same organism is present in each of these countries. All samples in the survey were tested with all four MPA probes and the HGC69a probe.

Competitor probe studies

When the competitor probes (Table 2) were employed, only filaments morphologically identified as "M. *parvicella*" bound to MPA650 thus overcoming the non-specificity of this probe. These two competitor probes each detected different types of cells when "M. *parvicella*" was present in samples. Probe CompMPA650.2 detected some *Streptomyces* species while probe CompMPA650.1 detected many members of the *Arthrobacter* line of descent in the high mol%G+C gram postives such as *Arthrobacter, Microbacterium, Aureobacterium* and *Cellulomonas* (Table 3). All of these latter genera are frequently isolated from sewage treatment plants (BARK et al., 1993; KÄMPFER et al., 1993) and in line with these results, CompMPA650.1 was able to detect many cells in activated sludge plants in our study. In addition, a set of 18 probes for the *Microbacterium* (two probes) and *Arthrobacter* groups (16 probes) in the high mol% G+C gram positives revealed a high abundance of these bacteria in activated sludge (ERHART, JUNKERMANN and AMANN, unpublished results). *In situ* typing of our activated sludges with these probes showed a marked diversity of hitherto unisolated or sequenced bacteria in this line of descent and confirmed the presence of the two groups mentioned above.

Staining of samples with fluorescent *in situ* hybridization probes and DAPI; intracellular inclusions and cellular rRNA content

A combination of fluorescent *in situ* hybridization probing and DAPI (1μg/ml) staining allowed *in situ* identification of "M. *parvicella*" filaments and also showed that these filaments often contain stainable polyphosphate inclusions. However, most segments of the filaments that contained large amounts of polyphosphates were low in ribosomes (Fig 1) and it may be concluded that the storage of polyphosphates could be a survival strategy for "M. *parvicella*".

A combination of DAPI and the REES (1992) method, or the Neisser staining method revealed that all cells in filaments morphologically identified as "M. *parvicella*" that contained polyhydroxy alkanoates or polyphosphate also contained DNA.

To study "M. *parvicella*" activity, we investigated two plants by counting 1000 filaments in each sample follow-

Table 3. Sequence alignments showing target sequences, matching target organisms and examples of non-target organisms for the MPA probes and their competitor probes. Not all target organisms are shown for the competitor probes. Capital letters indicate strong mismatches and small letters indicate weak mismatches according to STAHL and AMANN (1991). Stringency is defined as the formamide concentration necessary for complete discrimination of the reference strains. Organisms used for evaluation of probe specificities are printed in bold.

MPA60		Discriminating stringency (% formamide)
probe sequence (3'-5')	TCAGCTTGCGCCGGTAGG	
target sequence (5'-3')	AGUCGAACGCGGCCAUCC	
Microthrix parvicella	....	
Magnetic coccus	..g....G.........u	
Caedibacter caryophila	.........a..G...u.	
Dienococcus radiodurans	.........a..G...u.	
Sphingomonas parapaucimobilis	.........a....C.u.	20
Xanthomonas campestris	U.g....G.........N	
Azosprillum brasiliense	U.g....G..........	10
Marinococcus halophilus	......g.....Ga.g..	
Rhodopseudomonas marina	..g....G.......A.u	
Halovibrio variabilis	U.g....N......U..u	
Desulfovibrio gigas	U.g....G......U..u	

MPA645		Discriminating stringency (% formamide)
probe sequence (3'-5')	CGAGACTGATCTCAGGCC	
target sequence (5'-3')	GCUCUGACUAGAGUCCGG	
Microthrix parvicella	....	
Helicobacter pylori	........gg..A.....	
Haliscomenobacter hydrossis	.u..g..u.g.....u..	
Cellulomonas uda	.Gg...........G...	20
Frankia species	.Gg.A.g...........	

MPA223		Discriminating stringency (% formamide)
probe sequence (3'-5')	GATCCTCCCAGAGCGCCG	
target sequence (5'-3')	CUAGGAGGGUCUCGCGGC	
Microthrix parvicella	....	
Actinopolyspora mortivallis	..g......g........	
Hydrogenobacter thermophilus	ug.......g........	
Catenuloplanes japonicus	..g...U..g........	
Aureobacterium testaceum	..g...U..g........	
Cellulomonas turbata	..g...U..A........	10
Cellulomonas cellulans	.A...U...A........	15
Arthrobacter atrocyaneus	..U...U..g........	10
Corynebacterium aquaticum	.A....U..A........	15
Nocardioides albus	......U.Ug........	5
Brassica napus chloroplast	.g.......g......U.	
Pisum sativum chloroplast	.A.......g......U.	

MPA650		Discriminating stringency (% formamide)
probe sequence (3'-5')	CTGATCTCAGGCCATCCC	
target sequence (5'-3')	GACUAGAGUCCGGUAGGG	
Microthrix parvicella	....	
Frankia species	.g................	
Nanochlorum eucaryotum	.........u........	
Amycolatopsis azurae	.........u........	
Actinoplanes utahensis	.g.......u........	20
Streptomyces salmonis	.g.......u........	
Cellulomonas cellulans	.........G........	20
Microbacterium imperiale	.........G........	20
Agromyces ramosus	....g....G........	15
Actinomyces bovis	.g..N....G........	
Frankia species	.g...........C....	
Bifidobacterium bifidum	.g..g....G........	0
Micrococcus sedentarius	.....C...au.......	0
Terrebacter tumescens	.........Gu.......	10

CompMPA650.1		Discriminating stringency (% formamide)
probe sequence (3'-5')	CTGATCTCACGCCATCCC	
target sequence (5'-3')	GACUAGAGUGCGGUAGGG	
Microbacterium laevaniformans	....	
Microbacterium imperiale	....	
Cellulomonas flavigena	....	
Curtobacterium luteus	....	
Agromyces ramosus	....	
Thermocrispum agreste	....	
Dermobacter hominis	....	
Arthrobacter citreus	...........A......	20
Arthrobacter crystallopoietes	...........A......	20
Brevibacterium linens	...........A......	20
Microthrix parvicella	.........C........	
Microbacterium lacticum	................A.	n.d.[a]
Terrebacter tumescens	..........u.......	20
Actinomyces naeslundii	.g................	

CompMPA650.2		Discriminating stringency (% formamide)
probe sequence (3'-5')	CCGATCTCAAGCCATCCC	
target sequence (5'-3')	GGCUAGAGUUCGGUAGGG	
Streptomyces brasiliensis	....	
Streptomyces caelestis	....	
Streptomyces ambofaciens	....	
Streptomyces rimosus	....	
Actinoplanes philippinensis	....	
Catenuloplanes japonicus	....	
Actinomyces naeslundii	....	
Streptomyces bluensis	....	
Dermobacter hominis	.........g........	
Bifidobacterium bifidum	....g....g........	20
Microbacterium imperiale	.A.......g........	20
Streptomyces baldacii	.........gu.......	
Oscillatoria species	A.................	
Actinomyces multifermentans	....U....g........	
Microthrix parvicella	.A.......C........	15

[a] n.d. no discrimination possible even at higher stringency.

ing *in situ* hybridization and DAPI staining. In Willstaedt, Germany mixed liquor, 76% of the DAPI stainable *"M. parvicella"* filaments were EUB338 and MPA650 probe positive. In the foam of this plant, 62% of the DAPI stainable *"M. parvicella"* filaments were EUB338 and MPA650 probe positive. In Hirblingen, Germany, the comparative values for mixed liquor and foam were 89% and 55%, respectively. These results suggest that the activity of *"M. parvicella"* is reduced in part of the filaments in foams when compared with the activity of filaments in mixed liquor. Here, we interpret the actually measured rRNA content of the filaments as an indicator of metabolic activity or at least metabolic potential.

Discussion

"Microthrix parvicella" is recognized as one of the most important bulking and foaming bacteria in activat-

Table 4. Reference strains used for evaluation of the MPA probes.

Species	Source	Species	Source
Actinoplanes philippinensis	DSM 43019^T	*Cellulomonas uda*	LMG 16327^T
Agromyces ramosus	LMG 16680^T	*Corynebacterium aquaticum*	DSM 20146
Arthrobacter atrocyaneus	LMG 3814^T	*Curtobacterium luteum*	LMG 8786^T
Arthrobacter citreus	DSM 20133	*Microbacterium lacticum*	DSM 20427
Arthrobacter crystallopoites	DSM 20117	*Microbacterium laevanifomans*	LMG 3678^T
Aureobacterium barkeri	LMG 16341^T	*Microbacterium imperiale*	LMG 20530
Azospirillum brasilense	DSM 2293	*Nocardioides albus*	LMG 16212
Bifidobacterium bifidum	LMG 8810	*Sphingomonas parapaucimobilis*	LMG 10923^T
Brevibacterium linens	DSM 20425^T	*Terrabacter tumescens*	LMG 3818
Cellulomonas cellulans	DSM 20106	*Terrabacter tumescens*	LMG 16133
Cellulomonas turbata	LMG 4072^T		

Abbreviations: DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG – Laboratorium voor Microbiologie, Universiteit Ghent, Ghent, Belgium.

ed sludge plants (JENKINS et al., 1993). The design and evaluation of four different rRNA-directed *in situ* hybridization probes from the *"M. parvicella"* 16S rDNA sequence for the detection and identification of *"M. parvicella"* are reported here. Data presented show that each of the MPA probes specifically binds to activated sludge filaments that are morphologically identified as *"M. parvicella"* according to the keys presented in EIKELBOOM and VAN BUIJSEN (1981). The problem of entry of the probes into the *"Microthrix"* cells was successfully addressed by the use of mutanolysin which overcomes a major impediment in the use of fluorescent *in situ* hybridization probes with these organisms. Although lysozyme has been used for cell permeabilization prior to whole-cell probing for some gram positive bacteria (BEIMFOHR et al., 1993), we found mutanolysin gave superior results to lysozyme.

The MPA probes were developed from the 16S rDNA sequence of an Australian isolate of *"M. parvicella"* (X82546, BLACKALL et al., 1994). However, the activated sludge plants evaluated here come from a small range of countries of the world where this organism is commonly observed and the data show that at least in the plants evaluated, the organisms identified as *"M. parvicella"* are the same. Recently two more *"M. parvicella"* isolates have been obtained; one additional one from a different Australian sewage treatment plant and one from Italy (ROSSETTI et al., accepted). The 16S rDNA sequence of the Italian isolate is identical to that of the original isolate (ROSSETTI et al., accepted), while that for the second Australian isolate is nearly identical to the original isolate (BLACKALL et al., 1996). Our data extend the information obtained with the pure cultures and additionally, open the way for rapid evaluation of the global diversity of *"M. parvicella"* in sewage treatment plants. A more extensive survey of activated sludge plants with the MPA probes is required to confirm that all filaments identified as *"M. parvicella"* are the same. SODDELL et al. (1992) raised the question about the potential physiological differences between *"M. parvicella"* seen in different countries of the world. Our data show that by fluorescent *in situ* hybridization probing, the organisms are likely to be phylogenetically similar but further research is needed to address the physiological diversity of *"M. parvicella"*, an exercise which is currently hindered by the lack of suitable media for its cultivation.

WAGNER et al. (1994) reported the hybridization of *"M. parvicella"* with probe HGC69a but we were not able to confirm this. The method of cell permeabilization employed by WAGNER et al. (1994) was different to ours and could be an explanation for the discrepancy. However, it seems more likely that they misidentified the observed filaments which could possibly have been *Nostocoida limicola* I, since it is well recognised that the differentiation between this filament and *"M. parvicella"* in activated sludge samples is difficult (JENKINS et al., 1993). Our MPA probes will eliminate this and other confusions by facilitating unequivocal identification of *"M. parvicella"*. Theoretically, the HGC69a probe could be expected to bind to *"M. parvicella"* filaments because this organism is a member of the actinomycetes subphylum (BLACKALL et al., 1994). However, we now know that the 23S rRNA of *"M. parvicella"* has three mismatches to probe HGC69a in the target region, effectively preventing probe binding (unpublished data).

None of the four MPA probes bound to any of a wide range of pure cultures of gram positive and gram negative bacteria when they were tested by fluorescent *in situ* hybridization probing. Although some bacterial cells and filaments in activated sludge samples bound the MPA650 probe, when two competitor probes were employed, the non-specificity of this probe was eliminated. However, with four MPA probes to choose from in the determination of the diversity of *"M. parvicella"* throughout the world, the slight non-specificity of one probe is not seen as important, and the competitors can be employed if necessary.

The ability of *"M. parvicella"* to produce polyphosphate inclusions has been reported previously (EIKELBOOM, 1975; ROSETTI et al., accepted). We also found this and additionally, we provide evidence that suggests this storage polymer could be a survival strategy for *"M. par-*

vicella" cells. The pure culture of the Italian *"M. parvicella"* is also seen to accumulate polyphosphate in older, presumably stressed, cultures (ROSSETTI and TANDOI, personal communication). The anaerobic zone in phosphate removal plants could impose a stressful condition for *"M. parvicella"*, and induce this organism to store polyphosphate. Currently, there is no evidence concerning the ability of *"M. parvicella"* to release phosphate. However, this line of investigation should be followed.

Acknowledgements

The Confocal Microscope Facility of The University of Queensland was supported by a Large Equipment Grant from the Australian Research Council. Mr. COLIN MACQUEEN provided assistance with the laser scanning confocal microscope. The study was financed in part by agrant of the Deutsche Forschungsgemeinschaft to RA (Am 73/2-3).

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