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Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal

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Most filamentous bacteria in biological nutrient removal (BNR) processes have not been identified beyond their morphotype and simple staining reactions. Furthermore, the majority of sludge filaments observed under the microscope do not hybridize to commonly used phylogenetic probes for well characterized bacterial phyla such as the *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. Specific 16S rRNA-targeted oligonucleotide probes were designed for the phylum *Chloroflexi* (green non-sulfur bacteria) and optimized for use in fluorescence *in situ* hybridization. *Chloroflexi* have been implicated in BNR systems by phylogenetic identification of filamentous bacteria isolated by micromanipulation from sludge and culture-independent molecular phylogenetic surveys. The predominant morphotype responding to the probes was filamentous and these filaments were generally abundant in 10 Australian full-scale and two laboratory-scale BNR samples examined. Filamentous bacteria responding to a subdivision 1 *Chloroflexi* probe were rare in the samples, whereas subdivision 3 *Chloroflexi* filaments were very common in some sludges. This is in direct contrast to results obtained from molecular phylogenetic surveys of BNR systems where most sludge 16S rDNA clones belong to subdivision 1 and only a few to subdivision 3. It is suggested that filamentous bacteria belonging to the *Chloroflexi* phylum account for a large fraction of phylogenetically uncharacterized filaments in BNR systems and are likely to be abundant in such systems on a global scale.

Keywords: activated sludge, filamentous bacteria, fluorescence *in situ* hybridization (FISH), phylogeny, microbial ecology

INTRODUCTION

Filamentous micro-organisms are ubiquitous and conspicuous members of activated sludge wastewater treatment plant microbial communities (Seviour & Blackall, 1999). These micro-organisms traditionally have been identified by their morphology and simple staining reactions as described by Eikelboom & van Buijsen (1983) and Jenkins *et al.* (1993). The majority of filamentous bacteria in sludges, however, are still unidentified beyond these simple characteristics (Seviour & Blackall, 1999). Modification of activated sludge plants to include enhanced biological nutrient removal (BNR) favours growth of filamentous micro-organisms that can compete with floc-forming organisms, possibly leading to bulking and foaming problems (Eikelboom *et al.*, 1998). However, why filamentous micro-organisms are able to overgrow and outcompete floc-formers is still

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Abbreviations: BNR, biological nutrient removal; FISH, fluorescent *in situ* hybridization.

The EMBL accession numbers for the sequences reported in this paper are X84472 (strain SBR1029 16S rDNA), X84474 (strain SBR1031 16S rDNA), X84498 (strain SBR1064 16S rDNA), X84565 (strain SBR2022 16S rDNA), X84576 (strain SBR2037 16S rDNA) and X84607 (strain SBR2076 16S rDNA).

Table 1. Incidence of filamentous bacteria in a range of Australian biological wastewater treatment plants as determined by phase-contrast and FISH microscopy

Wastewater treatment plant*	Process type†	Abundance of filamentous organisms‡			
		General	*Chloroflexi*	Subdivision 1a,b	Subdivision 3
Oxley	C	4	1	Very few	None
Noosa 1	C, N	5	4	Few	Most
Noosa 2	C, N	4	4	Very few	Few
Noosa 3	C, N	4–5	4–5	Very few	Many
Gibson Island	C, N	6	4–5	Very few	Some
Murrumba Downs	C, N	5–6	4	Very few	Some
Luggage Point	C, N	6	4	Very few	Many
Wacol	C, N	3	1–2	Very few	Some
Brendale	C, N	4	3	Very few	Very few
Rouse Hill	C, N, P	4	3	Very few	Few
St Mary's	C, N, P	5	3	None	Few
Thornside	C, N, P	5	3	Very few	Some
Lab scale 1	C, P	2	1	Very few	Almost all
Lab scale 2	C, N	3	2	Few	None

* Rouse Hill and St Mary's are in New South Wales; the remainder of the plants are in Queensland. Noosa 1–3 indicates different sampling occasions (January, February and June).

† Type of biological wastewater treatment plant. C, organic carbon removal; N, nitrogen removal; P, phosphorus removal.

‡ Subjective scores of Jenkins *et al.* (1993) used for amount of general and *Chloroflexi* filaments are: 0, none; 1, few; 2, some; 3, common; 4, very common; 5, abundant; 6, excessive. The fractions of *Chloroflexi* filaments grouping in subdivision 1a, 1b and 3 as determined by subdivision-specific FISH are rated from none to all observed.

poorly understood (Seviour & Blackall, 1999). An improved understanding of the phylogeny and physiology of filamentous micro-organisms in BNR sludges is required for the formulation of effective bulking and foaming control measures (Seviour & Blackall, 1999).

Over the last decade, molecular biological methods have been used to identify and monitor filamentous micro-organisms (Blackall, 1994; Bradford *et al.*, 1996; Erhart *et al.*, 1997; Kanagawa *et al.*, 2000; Wagner *et al.*, 1994). In particular, fluorescence *in situ* hybridization (FISH) using 16S rRNA-targeting oligonucleotide probes (DeLong *et al.*, 1989) is an invaluable technique for directly identifying micro-organisms in their natural settings (Amann *et al.*, 2001). Thus far, only a minority of filamentous bacteria in BNR systems have been identified using phylogenetic stains (Seviour & Blackall, 1999). However, these comprise a wide diversity of organisms including members of well-studied bacterial phyla such as *Proteobacteria* (e.g. *Thiothrix* spp.; Howarth *et al.*, 1999; Kanagawa *et al.*, 2000), *Actinobacteria* (e.g. '*Microthrix parvicella*'; Blackall *et al.*, 1994), *Firmicutes* (e.g. some '*Nostocoida limicola*' I; Liu *et al.*, 2000) and *Bacteroidetes* (e.g. Type 0092, Bradford *et al.*, 1996). Recently, less studied bacterial phyla such as *Chloroflexi* (Beer *et al.*, 2002; Bradford *et al.*, 1996), *Planctomycetes* (Liu *et al.*, 2001) and candidate phylum TM7 (Hugenholtz *et al.*, 2001a) have also been shown by molecular methods to have filamentous representatives in sludge. Phylum-level FISH probes exist for the *Planctomycetes* (Neef *et al.*, 1998) and TM7 group (Hugenholtz *et al.*, 2001a), but only species-specific FISH probes have been published for the *Chloroflexi* phylum (Beer *et al.*, 2002; Sekiguchi *et al.*, 2001). Therefore, the extent of representatives belonging to this latter phylum in BNR sludges is unknown.

The aim of the present study was to design phylum- and subdivision-specific oligonucleotide probes for the *Chloroflexi* and to evaluate them on sludge samples using FISH, to determine the abundance, morphology and spatial distribution of *Chloroflexi* in activated sludges.

METHODS

Sample collection and processing. Activated sludge mixed liquor samples were collected from ten full-scale and two laboratory-scale biological wastewater treatment plants as listed in Table 1. One full-scale plant was sampled three times throughout the year (Noosa, Table 1). Aliquots of the sludge samples were fixed in paraformaldehyde and stored at −20 °C (Amann, 1995).

16S rDNA clone sequencing. The near complete 16S rDNA sequences were obtained for six clones from laboratory-scale BNR processes. These clones had previously been partially sequenced and placed in the *Chloroflexi* phylum (Bond *et al.*, 1995). DNA sequencing followed previously reported methods

Table 2. *Chloroflexi*-specific FISH probes and optimized conditions for use

For all optimization experiments 46 °C hybridization temperature and 48 °C washing temperature was used.

Oligonucleotide*	*E. coli* no.	Probe sequence (5′–3′)	Length (nt)	T_m (°C)†	G+C (%)	Optimal formamide concn (%)	Positive control	Negative control (no. mismatches)
GNSB-941	941–957	AAACCACACGCTCCGCT	17	60	59	35	*Herpetosiphon geysericola*	*Clostridium sporogenes* (1)
CFX1223	1223–1242	CCATTGTAGCGTGTGTGTMG	20	58	53	35	*Herpetosiphon aurantiacus*	*Escherichia coli* (2)‡
CFX109	109–126	CACGTGTTCCTCAGCCGT	18	61	61	30	*Herpetosiphon aurantiacus*	*Metallosphaera sedula* (1)
CFX784	784–801	ACCGGGGTCTCTAATCCC	18	59	61	35	Isolate UNI-1	*Planctomycetes* sp. (1)

* GNSB-941 was previously published by Gich *et al.* (2001); all other oligonucleotides were designed in this study.

† Determined by nearest-neighbour method and calculated using 50 mM NaCl and 50 μM oligonucleotide.

‡ A number of uncultivated TM7 bacteria have one mismatch to this probe (Hugenholtz *et al.*, 2001a).

(Bond *et al.*, 1995) except that the BigDye Terminator (Applied Biosystems) sequencing kit was used.

Phylogenetic analysis and probe design. *Chloroflexi* 16S rDNA sequences determined in the present study and available from the public databases were imported and aligned in the ARB software package (http://www.arb-home.de). Phylogenetic trees were inferred from the alignment as previously described (Klein *et al.*, 2001). The dataset was checked for chimaeric sequences by inferring independent trees from the 5′ and 3′ halves of the alignment and looking for branching incongruencies (partial treeing analysis). *Chloroflexi*-specific oligonucleotide probes were designed as described previously (Hugenholtz *et al.*, 2001a, b). Selected parameters of the probes are detailed in Table 2. Additionally, an oligonucleotide primer designed for *Chloroflexi*-specific PCR (Gich *et al.*, 2001) called GNSB-941f was evaluated unmodified as a FISH probe.

FISH microscopy and probe evaluation. Probes were commercially synthesized and 5′ labelled either with the fluorochrome fluorescein isothiocyanate (FITC) or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermohybaid Interactiva). The optimal stringencies of the probes were determined empirically using a previously reported method (Crocetti *et al.*, 2000). The pure cultures used as positive and negative controls are listed in Table 2. All negative controls had one central mismatch to the probes, except in the case of CFX1223 where organisms with one mismatch only exist as clones within the TM7 bacteria from sludge. Here, *Escherichia coli* with two mismatches to the probe was used as negative control. The 16S rDNAs of most control cultures were sequenced in the probe target sites using the same methods as used for sequencing clones (see above) to confirm that the target string had the expected sequence. In the case of *Metallosphaera sedula*, the FISH probe ARC915 (Stahl & Amann, 1991) was used to confirm the identity and purity of the culture.

FISH was carried out on paraformaldehyde-fixed samples with methods detailed by Amann (1995), using a 1·5 h hybridization time and published or determined formamide concentrations. Following FISH, samples were observed with a Bio-Rad Radiance 2000 confocal laser scanning microscope using a Nikon 60× oil immersion objective. FITC, Cy3 and Cy5 were excited with an Ar laser (488 nm), HeNe laser (543 nm) and red diode laser (637 nm) and collected with 500–530 nm BP, 550–625 nm BP and 660 LP emission filters, respectively. Images were collected and final image evaluation was done in Adobe Photoshop.

For the activated sludge screening, paraformaldehyde-fixed samples were triple hybridized with two *Chloroflexi* phylum or subdivision targeting probes and EUBMIX probe suite targeting most *Bacteria* (Daims *et al.*, 1999). CFXMIX was also used and this was composed of equal amounts of the *Chloroflexi* phylum probes GNSB-941 and CFX1223 labelled with the same fluorochrome.

Subjective scoring of abundance of filamentous bacteria and *Chloroflexi*. The abundance of all filamentous organisms and filamentous *Chloroflexi* in the samples was measured according to the subjective scoring method of Jenkins *et al.* (1993) where the observations are rated on a scale from 0 (none) to 6 (excessive) (Table 1). Phase-contrast images were captured on an Olympus BH2 microscope. Final image evaluation was done in Adobe Photoshop. *Chloroflexi*-specific measurements were made using Cy5 labelled CFXMIX (Table 1). Images were captured as previously mentioned. The proportions of filaments in *Chloroflexi* subdivisions 1 and 3 were determined using Cy3-labelled CFX784 or CFX109, respectively, in combination with Cy5-labelled CFXMIX. Filament abundances were again subjectively scored whereby the proportions of *Chloroflexi* 1 and 3 were ranked in relation to all *Chloroflexi*. Observations were rated from none to all observed (Table 1). All estimations were based on a mean of 7–10 independent hybridizations and subjective scorings.

RESULTS

Chloroflexi phylogeny

An evolutionary distance dendrogram of the *Chloroflexi* (green non-sulfur bacteria) phylum is presented in Fig. 1 based on comparative analyses of 16S rDNA sequences greater than 1300 nt long. Fig. 1 includes five SBR (sequencing batch reactor) clones fully sequenced for this analysis that were obtained in a previous study in which only partial sequences were determined (Bond *et al.*, 1995). One is in *Chloroflexi*-1a (SBR1029), two are in *Chloroflexi*-1b (SBR2037 and SBR1031), one (SBR2076) is in *Chloroflexi*-1 closely affiliated with another BNR clone (SBR1108, AF269004) and one (SBR2022) is in *Chloroflexi*-3 (Fig. 1). One further clone, SBR1064, proved to be chimaeric by partial treeing analysis, with an approximate breakpoint at *E. coli* position 1215. The 5′ fragment (1210 nt), belonging to

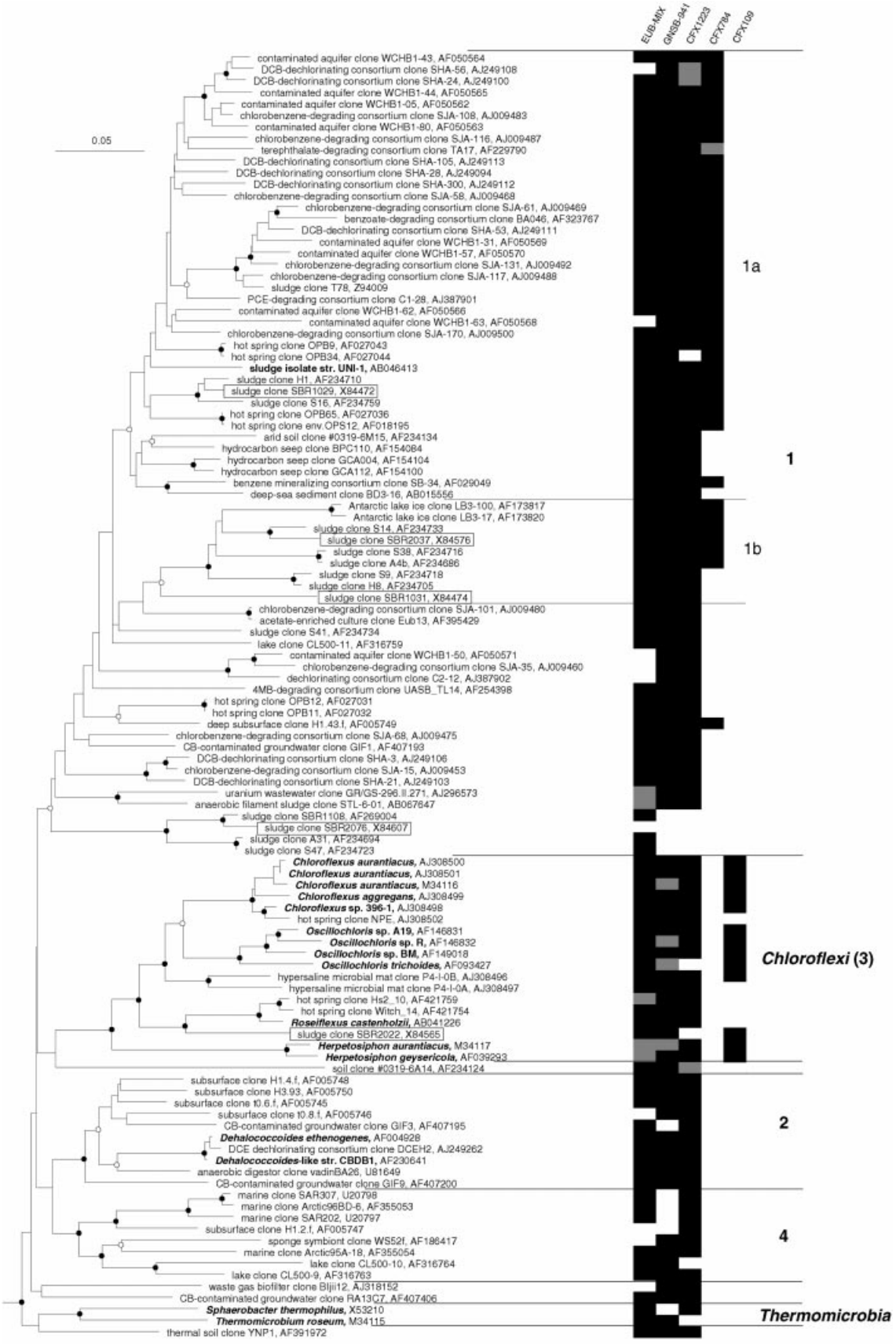

Fig. 1. For legend see facing page.

the *Chloroflexi* (99% identical to SBR1029), was submitted to the public databases and the 3′ fragment (225 nt), belonging to candidate phylum SBR1093 (99% identical to SBR1093, Hugenholtz *et al*., 2001a), was discarded. Subdivisions of the phylum were named according to an earlier phylogenetic analysis of the *Chloroflexi* (Hugenholtz *et al*., 1998). Monophyly of the subdivisions was confirmed by bootstrap resampling of the dataset using distance and parsimony inference methods under a variety of bacterial outgroup configurations (Dalevi *et al*., 2001). Most characterized isolates in the phylum are members of subdivision 3, whereas most environmental clone sequences belong to subdivision 1, a great number of which were obtained from pollutant-contaminated habitats (Fig. 1). A pure culture representative of subdivision 1 has recently been described as UNI-1 from a thermophilic, upflow anaerobic sludge blanket reactor (Sekiguchi *et al*., 2001). Two large reproducibly monophyletic clusters of sequences within subdivision 1, 1a and 1b, are indicated in Fig. 1. A number of environmental clone sequences (YNP1, RA13C7, Bljii12 and #0319-6A14) belonging to the *Chloroflexi*, are unaffiliated to subdivisions 1 to 4 and indicate the existence of additional subdivisions in the *Chloroflexi*.

Chloroflexi probe evaluation and application

Phylum- and subdivision-level probes were designed for *Chloroflexi* and evaluated with pure culture controls (Table 2). The two phylum-level probes, GNSB-941 and CFX1223, the subdivision 1(a, b) probe CFX784 and the subdivision 3 probe CFX109 were all successful in FISH and probe specificity was confirmed with negative controls. The optimized hybridization conditions for the probes are listed in Table 2. The determined optimal formamide concentrations for the probes are shown in Table 2. To facilitate rapid multiprobing, a value of 30% formamide was used for all probes throughout the sludge survey.

As with all broad-specificity probes it was difficult to design probes which hit all sequences in the target group. Compromises had to be made to obtain as broad a coverage of the *Chloroflexi* as possible while minimizing coverage of non-target organisms. This is highlighted in Fig. 1 where the specificity of the *Chloroflexi* probes and of the general bacterial probe, EUBMIX (Daims *et al*., 1999), is summarized in the columns to the right of the dendrogram. Probes with no mismatches to a given sequence in the dendrogram (likely to hybridize successfully to that sequence) are indicated by black squares and probes with one or more mismatches to a given sequence (unlikely to hybridize successfully to that sequence) are indicated by white squares. Grey squares indicate that the probe will likely hybridize to the target sequence, but that an unresolved base was present in the sequence. A grey square was also used in the case of *Herpetosiphon* and EUBMIX where there is one or more base mismatches between the probes and target string (Daims *et al*., 1999). Nevertheless, we found *Herpetosiphon* species successfully bound EUBMIX in FISH. The sequences targeted by the two phylum-level probes differed slightly (Fig. 1) and we recommend using the probes together as CFXMIX to improve overall coverage of the *Chloroflexi* phylum. The coverage of subdivision 1 by CFX784 was patchy; mainly 1a and 1b were targeted, including about half of the sludge-clone sequences. Subdivision 3 was well covered by CFX109 with the exception of *Roseiflexus* and very recently published *Roseiflexus*-like environmental sequences (Boomer *et al*., 2002).

BNR sludge survey

All full-scale sludge samples investigated had common to excessive general filamentous bacterial populations (Table 1). In the two laboratory-scale sludges the number of filaments was lower than in the full-scale sludges (Table 1). *Chloroflexi* were ubiquitous in the samples examined by FISH (Table 1) and the predominant morphotype observed was filamentous, suggesting that this morphotype is common and widespread in the *Chloroflexi*. In six of 12 full-scale plant samples examined, *Chloroflexi* were ranked as very common to abundant and in only one full-scale plant were *Chloroflexi* ranked at few (Table 1). The fraction of subdivision 1 and 3 *Chloroflexi* filaments relative to all *Chloroflexi* filaments was subjectively scored and the results from many observations are recorded in Table 1. Images representing particular results are shown in Fig. 2.

In some samples, nearly all filaments bound both phylum-specific probes (GNSB-941 and CFX1223) and EUBMIX as shown by white filaments in Fig. 2(a). However, numerous instances of filaments binding only one of the phylum-specific probes were observed. Fig. 2 (b, c) shows examples of where most *Chloroflexi* are magenta due to binding EUBMIX and GNSB-941 but not CFX1223. Cells binding CFX1223 but not GNSB-941 were rarely observed. Some white *Chloroflexi* filaments binding EUBMIX, GNSB-941 and CFX1223 are also visible in Fig. 2(b, c). In several cases, *Chloroflexi* did not hybridize to the general bacterial

Fig. 1. Evolutionary distance dendrogram of the bacterial phylum *Chloroflexi* based on comparative analysis of 16S rDNA data. Sequences from isolates are shown in bold. SBR clones fully sequenced in the present study are boxed. Branch points supported by bootstrap resampling are indicated by black circles (bootstrap proportion values >90%) and white circles (bootstrap proportion values >75%). Representative sequences of the OP9 phylum were used as outgroups (not shown). The scale bar indicates 0·05 changes per nucleotide. Probe specificities are shown to the right of the figure as are the subdivision affiliations of the sequences. Black squares indicate a perfect match between the probe and target sequences, white squares indicate one or more mismatches between the probe and target sequences and grey squares indicate unresolved bases in the target sequence which are likely to match the probe sequence.

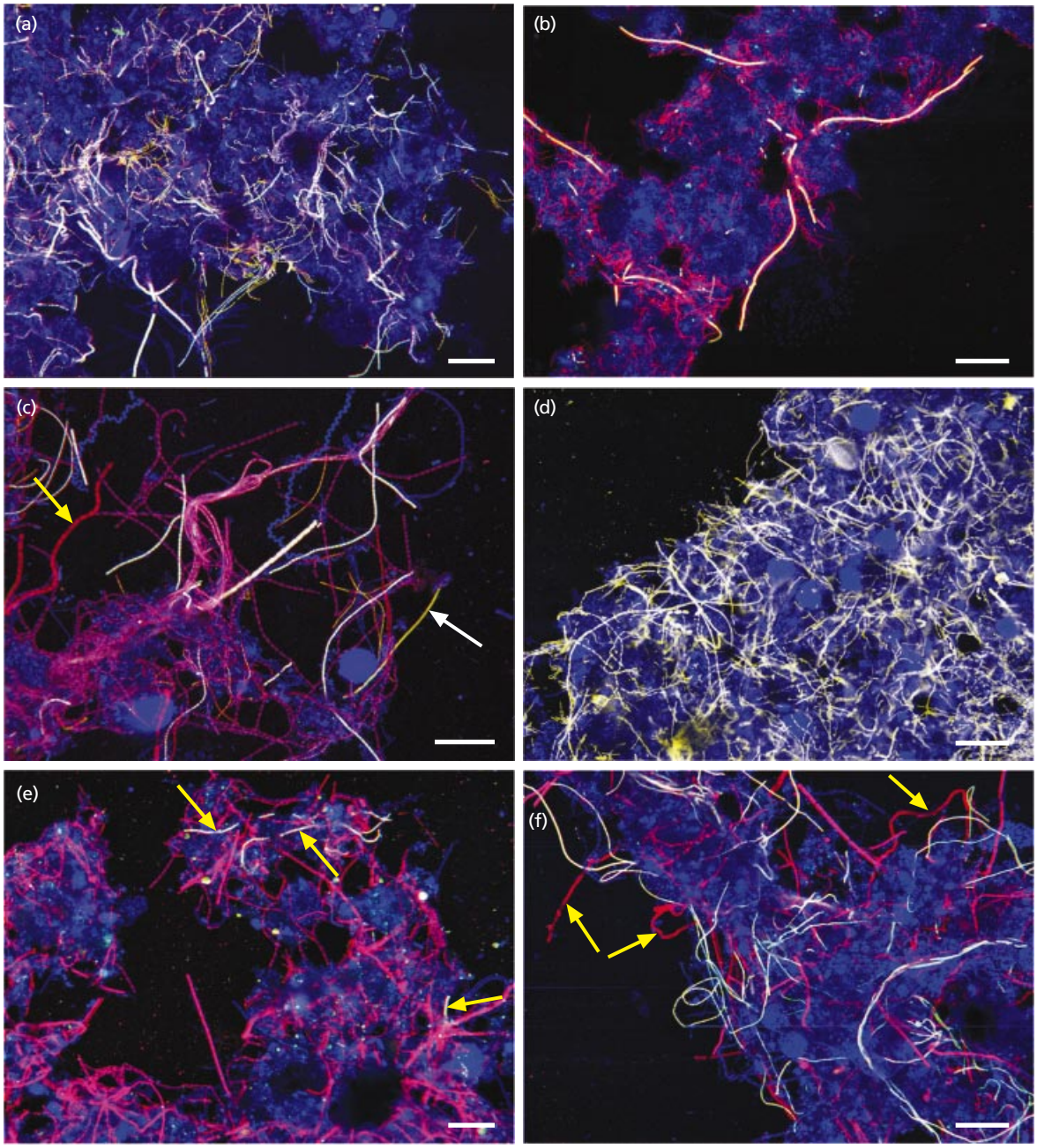

Fig. 2. Confocal laser scanning micrographs of FISH of selected BNR sludge samples. In all cases, the colours of the different probes are indicated in parentheses after the probe. In superimposed images, the overlap between red and green is yellow, between red and blue is magenta, and between red, green and blue is white. All bars are 20 μm. (a) Rouse Hill. The two phylum probes show good overlap and cells responding to EUBMIX (blue), CFX1223 (green) and GNSB-941 (red) are shown as white. (b) Lab scale 2. The numbers of filaments responding to EUBMIX (blue) and GNSB-941 (red) are high (magenta) whereas only a few larger white filaments are also binding CFX1223 (green). (c) Gibson Island. Filaments binding both *Chloroflexi* phylum probes GNSB-941 (red) and CFX1223 (green) but not EUBMIX (blue) appear yellow (white arrow). Some filaments are red (yellow arrow) due to binding GNSB-941 (red) but not EUBMIX (blue). (d) Noosa 3. *Chloroflexi* binding EUBMIX (blue) and CFXMIX (both red and green) are shown as white filaments inside the floc. (e) Noosa 3. White filaments (arrowed) are *Chloroflexi*-1 due to binding EUBMIX (blue), CFXMIX (red) and CFX784 (green), while all other *Chloroflexi* appear magenta (EUBMIX and CFXMIX). (f) Luggage Point. White filaments are *Chloroflexi*-3 due to binding EUBMIX (blue), CFXMIX (red) and CFX109 (green), some *Chloroflexi* appear magenta (EUBMIX and CFXMIX) and some are red (arrowed) due to binding only CFXMIX (red).

probe EUBMIX and consequently appeared red due to binding only GNSB-941 or yellow due to binding both phylum-level probes (Fig. 2c). All possible combinations of EUBMIX, GNSB-941 and CFX1223 were expected based on the probe specificities of *Chloroflexi* sequences (Fig. 1).

The maximum intended target group could be observed by the use of the two phylum-specific probes (GNSB-941 and CFX1223) in a mixture called CFXMIX. FISH using CFXMIX and EUBMIX showed that the presence of filamentous *Chloroflexi* was in general very high (e.g. Fig. 2d). As reflected in the selection of images shown in Fig. 2, the filamentous *Chloroflexi* commonly occurred inside flocs.

Of the subdivisions investigated, *Chloroflexi*-1 was less abundant than *Chloroflexi*-3 (Table 1). This is demonstrated by the relatively low abundance of white filaments in Fig. 2(e) (*Chloroflexi*-1) and greater abundance of white filaments in Fig. 2(f) (*Chloroflexi*-3). *Chloroflexi*-1 were generally thin (<1 μm), medium length (10–50 μm), smooth filaments mainly found inside bacterial flocs but were occasionally thick (>1 μm), short (<10 μm), segmented filaments bridging flocs. There were several different morphotypes of *Chloroflexi*-3 including thin, short and long (>50 μm) intrafloc filaments; thick curved filaments; straight, thick (>2 μm) filaments; segmented, thin and thick (>2 μm), long, interfloc-bridging filaments; and thin filaments arranged in bundles and composed of clearly demarcated cells giving the impression of beads.

In the case where a full-scale plant (Noosa) was sampled on several different occasions, the total number of filamentous bacteria and *Chloroflexi* observed was fairly constant. However, between different Noosa samples, there was great variation in *Chloroflexi*-3 abundance, ranging from most of the *Chloroflexi* to only a few (Noosa 1-3, Table 1).

DISCUSSION

We successfully designed and evaluated a suite of FISH probes for the phylum *Chloroflexi* (probe CFX1223) and for its two largest subdivisions (CFX784 for subdivision 1 and CFX109 for subdivision 3). Additionally, the oligonucleotide GNSB-941, designed for use as a *Chloroflexi* phylum PCR primer (Gich *et al*., 2001) was demonstrated to be directly applicable as a FISH probe with a centralized mismatch to most non-target 16S rRNAs. Using these probes, we demonstrated that filamentous representatives of the phylum *Chloroflexi* are generally abundant in BNR activated sludge biomass. In this study, simple subjective filament scoring was satisfactory for the overall filament abundances, but it should be recognized that digital image analysis methods are available for definitive microbial quantification.

Bergey's Manual of Systematic Bacteriology has formally proposed the name *Chloroflexi* (Garrity & Holt, 2001) to supersede the previous common name 'green non-sulfur' (Woese, 1987) for this phylum of bacteria. *Chloroflexi* comprises four well-represented subdivisions labelled 1 to 4 in Fig. 1 in accordance with a previous classification of this phylum (Hugenholtz *et al*., 1998). *Chloroflexi*-1 has recently undergone significant expansion due to the addition of many environmental clone sequences, and within this subdivision, there are two large monophyletic groups we have called a and b (Fig. 1) which are relatively well-targeted by CFX784. The environmental clone sequences of *Chloroflexi*-1 largely come from pollutant-contaminated habitats, while the only pure culture, UNI-1, is from a recently described upflow anaerobic sludge blanket reactor (Sekiguchi *et al*., 2001). *Chloroflexi*-2 contains the well-known tetrachloroethene dechlorinator *Dehalococcoides ethenogenes* (Maymó-Gatell *et al*., 1997) along with clone sequences (Fig. 1). No target sites suitable for a *Chloroflexi*-2 probe were found. *Chloroflexi*-3 contains most of the pure-cultured representatives of *Chloroflexi* including *Chloroflexus* spp., *Oscillochloris* spp., *Roseiflexus castenholzii*, *Herpetosiphon* spp. and *Heliothrix oregonensis*, the last of which is not shown in Fig. 1 due to only a partial 16S rDNA sequence (871 nt) being available in the public databases. The sequences in *Chloroflexi*-3 are relatively well targeted by CFX109. This subdivision is formally proposed as the class *Chloroflexi* (Garrity & Holt, 2001). *Chloroflexi*-4 is composed of clone sequences from marine and lake-water environments and, as was the case for subdivision 2, no suitable probe sites were found for this subdivision. In addition to the four well-represented subdivisions, there are at least five further subdivision-level lineages as evidenced by one or a few 16S rDNA sequences for each lineage. These include *Thermomicrobium roseum* and *Sphaerobacter thermophilus* which form a monophyletic subdivision in the *Chloroflexi* (*Thermomicrobia* in Fig. 1). These organisms were originally included in subdivision 3 (Hugenholtz *et al*., 1998), but this relationship has not held up with the inclusion of additional sequences (Fig. 1). Furthermore, *T. roseum* is classified in a separate phylum, the *Thermomicrobia*, in Bergey's Manual (Garrity & Holt, 2001) and *S. thermophilus* as a member of the *Actinobacteria* (Demharter *et al*., 1989; Garrity & Holt, 2001). However, present phylogenetic evidence indicates that both are members of the *Chloroflexi* phylum (Fig. 1).

Chloroflexi-1 contains the majority of 16S rDNA sequences from molecular phylogenetic surveys including 16 of the 17 clones obtained from activated sludge studies shown in Fig. 1. The majority of activated sludge clones in *Chloroflexi*-1 (11 clones of 16) were generated from full-scale activated sludge biomass (Juretschko *et al*., 2002; Snaidr *et al*., 1997). However, using FISH, *Chloroflexi*-3 filaments in full-scale activated sludge processes were more abundant than *Chloroflexi*-1 filaments (Table 1). This discrepancy is most likely explained by the non-quantitative nature of PCR-clone libraries which can give skewed representations of the relative abundance of organisms present in a sample largely due to the PCR step (Hugenholtz & Goebel,

2001). *Chloroflexi*-3 is best known from hotspring and hypersaline isolates or clones but does contain isolates such as *Herpetosiphon* species obtained from full-scale activated sludges (Bradford *et al*., 1996; Senghas & Lingens, 1985; Trick & Lingens, 1984) and a clone from a laboratory-scale process (SBR2022, Bond *et al*., 1995). *Herpetosiphon* was found to be responsible for bulking in this environment and its role in degradation of macromolecules from influent sewage was speculated upon (Reichenbach, 1992). Recently, an isolate of another filamentous bulking sludge organism, Type 1851 was phylogenetically placed within *Chloroflexi*-3, most closely related to *R. castenholzii* but with only 84% identity (Beer *et al*., 2002). In our survey, some of the sludge filaments binding the *Chloroflexi*-3 probe CFX109 could have been *Herpetosiphon* sp. or Type 1851. However, their common location buried within sludge flocs, precluded us from using their morphology as observed by phase-contrast microscopy or by staining and bright-field microscopy to identify them. By FISH, the filamentous bacteria were highly visible even when present in the centre of flocs, and clearly identifiable due to the phylogenetic basis of the oligonucleotide probe design (see Fig. 2). This demonstrates the advantages of FISH over the traditional *in situ* identification method. The application of FISH would also reduce underestimation of filamentous bacteria when they occur within sludge flocs.

Molecular phylogenetic surveys indicate that members of the *Chloroflexi* are found in numerous diverse habitats apart from activated sludges, such as geothermal springs (Boomer *et al*., 2002); hypersaline mats (Nübel *et al*., 2001); the deep subsurface (Chandler *et al*., 1998); and aerobic/anoxic (Juretschko *et al*., 2002), anaerobic (Sekiguchi *et al*., 2001) and dechlorinating enrichments (Maymó-Gatell *et al*., 1997). Isolated representatives of the *Chloroflexi* display a wide range of phenotypes (Hugenholtz *et al*., 1998). However, from our study, we cannot deduce the physiological traits of the *Chloroflexi* filaments in sludge. For example, most of the isolated bacteria belonging to *Chloroflexi*-3 are phototrophic, but we cannot infer phototrophy for filaments binding the *Chloroflexi*-3 probe (CFX109) because the target group is too broad and likely contains representatives with a wide range of physiologies. *Chloroflexi*-specific FISH using the probes described in this study could be used in concert with *in situ* microautoradiography to determine specific aspects of their phenotype (Lee *et al*., 1999) or be used to direct cultivation attempts.

The investigated sludges were not suffering from bulking and the general intrafloc location of the *Chloroflexi* would not support a potential role in bulking. Bossier & Verstraete (1996) suggest filamentous organisms in activated sludge provide a stabilizing backbone for the three-dimensional microbial aggregates called flocs. The intrafloc location of the *Chloroflexi* and their relative abundance support this hypothesis which likely explains one important role for these organisms in the activated sludge ecosystem. The potential role of *Chloroflexi* in macromolecule degradation (Reichenbach, 1992) should also be evaluated. Therefore, collectively, there appear numerous important roles for *Chloroflexi* in activated sludge and these roles have not been well studied in relation to *Chloroflexi* microbial ecology.

Although only sludges from Queensland and New South Wales were inspected for *Chloroflexi* in the present study, we anticipate that representatives of this bacterial phylum will be ubiquitous in activated sludges because the microbial communities in activated sludge demonstrate a remarkable consistency on a global scale (Seviour & Blackall, 1999). For example, the important bulking filament '*M. parvicella*' was initially phylogenetically characterized from an Australian isolate (Blackall *et al*., 1994) which subsequently proved to be representative of these organisms in activated sludge globally. The probes designed and optimized in this study will likely be useful in the study of wastewater treatment and in microbial ecology studies in general.

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