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Filamentous Bacterium Eikelboom Type 0092 in Activated Sludge Plants in Australia Is a Member of the Phylum *Chloroflexi*▽

Lachlan Speirs, Tadashi Nittami, Simon McIlroy, Sarah Schroeder, and Robert J. Seviour*

Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3552, Australia

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Molecular data show that the filamentous bacterium Eikelboom type 0092, frequently seen in Australian activated sludge plants, is a member of the phylum *Chloroflexi*. Fluorescence in situ hybridization (FISH) probes designed against cloned 16S rRNA sequences from a full-scale enhanced biological phosphate removal-activated sludge plant community, where this was a dominant filament morphotype, suggest that it can exist as two variants, differing in their trichome diameter. When applied to samples from several treatment plants in eastern Australia, each FISH probe targeted only the type 0092 filament morphotype against which it was designed. The patterns of FISH signals generated with both were consistent with the ribosomes not being evenly distributed but arranged as intracellular aggregates. The FISH survey data showed that these two variants appeared together in most but not all of the plants examined. None stained positively for intracellular presence of either poly-β-hydroxyalkanoates or polyphosphate.

Most activated sludge plants suffer from the operational disorders of bulking and foaming, both of which are caused by excessive growth of certain filamentous bacteria. Several different filament morphotypes have been described from systems treating domestic and industrial wastes (17, 18) but, in the absence of pure cultures, many of these have never been characterized sufficiently to resolve their taxonomy or provide them with valid names. Hence, they are often still referred to as numerical types persisting from the study of Eikelboom (18). Success has been achieved with some cultured and uncultured filaments in elucidating their phylogeny from 16S rRNA sequence analyses (6, 9, 10, 31, 50) and providing them with valid names (37, 49). Furthermore, with such sequence information, rRNA targeted oligonucleotide probes have been designed for their in situ identification and, together with microautoradiography (MAR) and other techniques (29, 40), their ecophysiology may be elucidated (25, 27, 28).

Type 0092, originally described by Eikelboom (18), appears prominently in many filament surveys carried out on plants around the world, where microscopy was used to identify them (35, 48). These morphotypes have been associated especially with long sludge age (>15-day) operational conditions (22) and thus frequently appear in enhanced biological phosphate removal (EBPR) systems (see, for example, reference 11), where the biomass is recycled repeatedly through anaerobic: aerobic zones. Consequently, this filament morphotype was classified as an "all-zone" grower by Wanner and Grau (52), able in their view to grow under aerobic, anoxic, and anaerobic conditions. However, its physiology from pure culture studies was described as being strictly aerobic (13, 21). These isolates were never deposited in recognized culture collections, and so confirmation of their identity is difficult. Similarly, the precise identification of the type 0092 filaments claimed to have been cultured by Ramothokang et al. (43) is unclear.

Type 0092 has very distinctive morphological features and so can be readily "identified" microscopically by its positive Neisser staining reaction and its short blunt-ended trichomes extending from the flocs or suspended in the bulk liquid (22). On the basis of 16S rRNA sequenced micromanipulated cultures, Bradford et al. (12) suggested it was a member of the *Bacteroidetes*, and yet filaments with the morphological features of type 0092 never fluoresced in situ with 16S rRNA targeted fluorescence in situ hybridization (FISH) probes designed against this sequence (37; E. M. Seviour, unpublished data). Consequently, it now seems unlikely that the organisms that grew from the micromanipulated filament with the type 0092 morphotype are type 0092 but rather are contaminating faster-growing filaments.

Regular microscopic examination of an EBPR full scale plant in Bendigo, Victoria, Australia, revealed that the biomass was always heavily dominated by a Neisser-positive filament fitting the microscopic description of type 0092 (see Fig. 1a). Therefore, attempts were made with FISH and 16S rRNA clone library construction to determine whether this filament morphotype could identified. We describe it here as a member of the phylum *Chloroflexi* and detail FISH probes designed for its in situ identification.

MATERIALS AND METHODS

Examination of biomass samples. Biomass samples were collected from 17 full-scale plants from eastern states in Australia and fixed in 4% (wt/vol) paraformaldehyde for FISH (2). Neisser staining was carried out as described by Jenkins et al. (22) on unfixed samples to determine whether they contained filamentous bacteria with the diagnostic morphological and staining properties of type 0092 filaments.

Preparation of 16S rRNA clone library. The DNA was extracted from a fresh sample of biomass from the Bendigo (Victoria, Australia) full-scale modified University of Cape Town EBPR plant which contained type 0092 as a dominant filament. Three different DNA extraction methods were used in attempts to minimize any potential biases associated with each, and the extracts were combined. These three methods included those of McVeigh et al. (38) and McIlroy et al. (36) and a FASTDNA spin kit (Qbiogene, Melbourne, Australia). The

* Corresponding author. Mailing address: Biotechnology Research Centre, La Trobe University, P.O. Box 199, Bendigo, Victoria 3552, Australia. Phone: 61 3 5444 7456. Fax: 61 3 5444 7476. E-mail: r.seviour@latrobe.edu.au.

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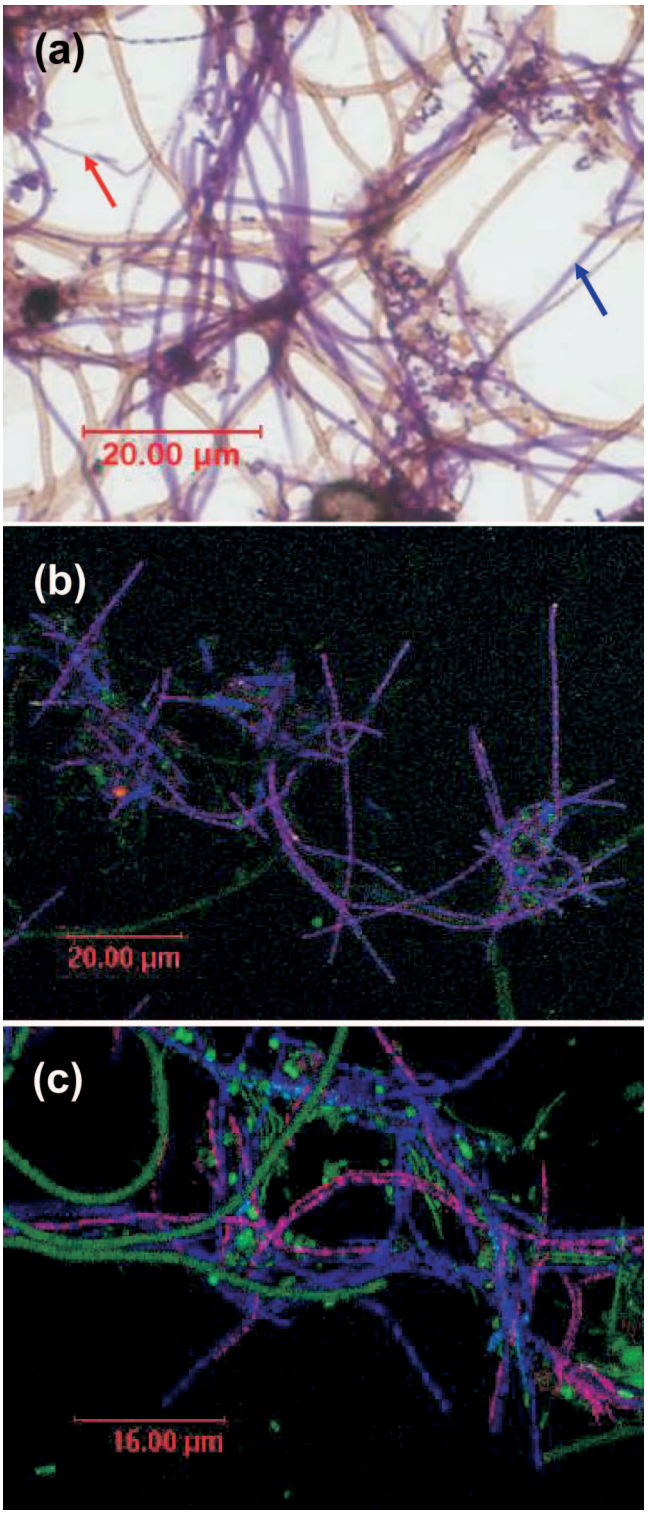

FIG. 1. (a) Neisser-stained biomass from the Bendigo wastewater treatment plant showing Neisser-positive type 0092 filaments. Blue arrow = thicker type 0092; red arrow = thinner type 0092 filaments. (b) Confocal laser-scanning microscopy image of FISH-probed biomass from the Bendigo EBPR plant. Magenta cells are those responding to both GNSB941/CFX1223 mix (CY5) and CFX197 (CY3) FISH probes for *Chloroflexi* type 0092. Note the localized fluorescence signal. Blue cells are those responding to the GNSB941/CFX1223 mix probes alone. Note the absence of EUBmix FISH target sites; green cells are those responding only to the EUBmix (FLUOS) probes; light blue cells are those responding to EUBmix plus GNSB941/CFX1223 mix probes together. (c) Confocal laser-scanning microscopy image of FISH probed biomass from the Bendigo EBPR plant. Magenta cells are those responding to both GNSB941/CFX1223 mix (CY5) and CFX223 (CY3) FISH probes for *Chloroflexi* type 0092. Note again the localized fluorescence signal. Blue cells are those responding to the GNSB941/CFX1223 mix probes alone. Blue and magenta cells lack EUBmix FISH target sites; green cells are those responding only to the EUBmix (FLUOS) probes; light blue cells are those responding to EUBmix plus GNSB941/CFX1223 mix probes together.

former two were selected because they had performed best in comparative trials at recovering DNA from marker activated sludge populations known to resist many DNA extraction protocols (S. McIlroy, K. Porter, S. Schroeder, R. J. Seviour, and D. Tillett, unpublished data).

Five PCRs were performed on the DNA from each extraction method, and all of the resulting PCR products were pooled to minimize any PCR-associated biases, using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). The 16S rRNA gene sequences were amplified with the primers U27f (5′-GAGTTTGATCMTGGCTCAG-3′) and U1492r (5′-GGYTACCTTGTTACGACTT-3′) under the following PCR conditions: 1 cycle of 10 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C; and 1 cycle of 10 min at 72°C. Each PCR mixture (50 μl) contained 1 μl of template DNA, 0.2 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 5 μl of 10× PCR buffer, 2.5 mM $MgCl_2$, and 1.25 U of AmpliTaq Gold (Applied Biosystems). All of the amplified PCR products were then combined and run on 1.5% agarose gels. Bands of ~1,450 bp were excised with a clean razor and purified with the Wizard SV gel and PCR clean-up system (Promega, Melbourne, Australia) according to the manufacturer's instructions.

Clone library construction. Purified PCR products were cloned into the pGEM-T Easy vector system (Promega). The presence of correctly sized inserts was checked by agarose gel electrophoresis of the clone colony PCR products using the PCR conditions described above. Plasmids from each clone were extracted with the Wizard Plus SV Minipreps DNA purification system (Promega). Initially, partial sequencing (approximately the first 500 bp) of the inserts was carried out by AGRF, Brisbane, Australia, and clones of interest were selected for complete sequencing based on the presence or absence of particular signature sequences of interest, as detailed below in Results. Possible chimeric sequences were assessed by using Bellerophon v3 (16), Mallard (5), and Pintail (4) software, and all putative chimeras were eliminated from subsequent analyses. The remainder were added to ARB (32) and aligned. A maximum-likelihood phylogenetic tree was constructed from these and selected related sequences.

FISH analyses. FISH was performed on biomass samples according to the protocol of Amann (2). The probes used are listed in Table 1, and the hybridization conditions applied were those detailed in the original publications for each. FISH probes developed during the present study were designed with the ARB software package (32). They were validated against biomass samples by incrementally increasing the formamide concentrations until only filaments with the desired morphotype fluoresced and before their fluorescent signal strength began to decrease. All probes were purchased from Proligo (Melbourne, Victoria, Australia) and were fluorescently tagged with CY3 and CY5 fluorochromes as detailed in Results.

Staining. To detect polyphosphate (polyP) and poly-β-hydroxyalkanoates (PHA) in cells, DAPI (4′,6′-diamidino-2-phenylindole) (23) and Nile blue A (42), respectively, were used as detailed in Ahn et al. (1).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB445103 to AB445106.

RESULTS

Identification of type 0092. Most of the biomass samples taken from Australian EBPR and non-EBPR plants located in three eastern states (Table 2) contained Neisser-positive short filaments extending from the floc into the bulk liquid or, occasionally, freely suspended in the bulk liquid. These fitted the

TABLE 1. FISH probes used in this study and their hybridization conditions

Probe	Target	Sequence (5′–3′)	Formamide (%)	Source or reference
EUB338 I[b]	All *Bacteria*	GCTGCCTCCCGTAGGAGT	0–50	3
EUB338 II[b]	*Planctomycetales*	GCAGCCACCCGTAGGTGT	0–50	14
EUB338 III[b]	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	0–50	14
HHY	*Haliscomenobacter hydrossis*	GCCTACCTCAACCTGATT	20	51
BAC303	*Bacteroidetes*	CCAATGTGGGGGACCTT	0	33
PLA886	*Planctomycetales*	GCCTTGCGACCATACTCCC	35	39
BET42A	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	35	34
CFB719	*Cytophaga-Flavobacterium-Bacteroides*	AGCTGCCTTCGCAATCGG	30	53
CF319a	Flavobacteria; some *Bacteroidetes*; some sphingobacteria	TGGTCCGTGTCTCAGTAC	35	33
CHL1851	Type 1851 filamentous bacterium	AATTCCACAACCTCTCCA	35	6
CFX109	*Chloroflexi* subgroup 3	CACGTGTTCCTCAGCCGT	30	8
CFX784	*Chloroflexi* subgroup 1	ACCGGGGTCTCTAATCCC	35	8
CFX1223	All *Chloroflexi*	CCATTGTAGCGTGTGTGTMG	35	8
GNSB941	All *Chloroflexi*	AAACCACACGCTCCGCT	35	20
CFX197	*Chloroflexi* OTU A (clones A26, B1, and B45) (variant A)	TCCCGGAGCGCCTGAACT	40	This study
CFX197comp	Competitor probe against sequences with accession no. ZA3635c and ZA3612c	TCCCGAAGCGCCTGAACT[a]		This study
CFX223	*Chloroflexi* OTU B (clone A58) (variant B)	GGTGCTGGCTCCTCCCAG	35	This study
CFX223 H202	Helper probe	AGCGCCTGAGCTTCAGTCATC		This study
CFX223 H241	Helper probe	CGTTACCTTACCAACTAGCTGATGG		This study

[a] The mismatching base of the CFX197comp probe to use with the CFX197 probe is underlined.
[b] EUB338 I, II, and III used in equimolar amounts as EUBmix.

morphological description of type 0092 (Fig. 1a). FISH analyses of biomass from the Bendigo plant showed that filaments with this distinctive morphology failed to respond to the EUB338 I, II, and III probes designed to target all *Bacteria* (14), regardless of whether these were applied individually or in combination. Of the other FISH probes tested (Table 1), this filament morphotype fluoresced only with the GNSB941 and CFX1223 probes designed to target members of the *Chloroflexi* (Fig. 1b). Another slightly thinner Neisser-positive filament (trichome ~0.67 μm in diameter compared to ~0.80 μm) also fluoresced with these two probes (Fig. 1c) and, in each case, the fluorescent signal generated was unevenly distributed and granular in appearance. Neither of these two Neisser-positive filament variants responded to the CHL1851 or CFX109 and CFX784 probes targeting Eikelboom type 1851 and the subgroups 3 and 1a of the *Chloroflexi*, respectively (Table 1). Also present in this sample were Neisser-negative filaments more similar in appearance to type 0092 than to the much thinner *Haliscomenobacter hydrossis*.

TABLE 2. Results of FISH-based survey of Australian EBPR and non-EBPR full-scale plants for type 0092 using the CFX197 and CFX223 probes

Biomass sample[a]	Filament count[b]		
	Neisser staining	CFX197	CFX223
Bendigo (VIC)	6	5	3
Logan (QLD)	4	4	3
Thorneside (QLD)	2	2	1
Coolum (QLD)	3	3	2
Nambour (QLD)	3	3	2
Merrimac (QLD)	5	3	2
Kyneton (VIC)	1	1	0
Castlemaine (VIC)	1	1	0
Watella (QLD)	5	5	3
Mornington (VIC)[c]	3	3	0
Daylesford (VIC)[c]	2	2	0
Carrum (VIC)	0	0	0
Maroochydore (QLD)	2	2	1
Dalby (QLD)	5	4	3
Morpeth (NSW)	2	3	1
Orange (NSW)	3	3	0

[a] VIC, Victoria, Australia; QLD, Queensland, Australia; NSW, New South Wales, Australia.
[b] Grading was as described in Jenkins et al. (22): 1, few; 2, some; 3, common; 4, very common; 5, abundant; and 6, excessive. The uptake of Neisser stain by floc EPS material meant the visualization of filaments within flocs was difficult. Therefore, estimations based only on staining may underestimate type 0092 abundance.
[c] Non-EBPR plant.

Design of FISH probes against *Chloroflexi* filaments. A library of 75 partial 16S rRNA gene sequences generated from the Bendigo EBPR plant biomass contained 11 *Chloroflexi* clones lacking the EUBmix signature probe target sites (Table 3). Representatives of the four OTUs they formed (based on shared 99% similarities) were then fully sequenced. Clone A58 is representative of an OTU of six clones, clones B1 and B54 each represent an OTU containing two clones, and A26 represents an OTU of a single clone. They formed two clusters, A (clones A26, B1, and B45) and cluster B (clone A58) in the phylogenetic tree (Fig. 2). All of these sequences also lacked the CHL1851, CFX109, and CFX784 target site sequences but contained those for the GNSB941 and CFX1223 probes (Table 1), which increased confidence in them being derived from the two filament morphotype variants suggested from the FISH data described above.

Two FISH probes were then designed against their sequences, CFX197 and CFX223, with the former designed to target the *Chloroflexi* clones in cluster A (Fig. 1b), and the latter the single clone A58 in cluster B (Fig. 1c). Their sequences are given in Table 1. A competitor probe

TABLE 3. FISH probes target site mismatches used to screen *Chloroflexi* 16S rRNA clone sequences to identify tentatively those derived from type 0092, as assessed by FISH analysis of Bendigo activated sludge biomass

Probe(s)	16S rRNA target sequence[a]	Reference
EUB338 I	ACTCCTACGGGAGGCAGC	3
Clones A26, A58, B1, and B45	ATACCTACGGGTAGCAGC	
EUB338 II	ACACCTACGGGTGGCTGC	14
Clones A26, A58, B1, and B45	ATACCTACGGGTAGCAGC	
EUB338 III	ACACCTACGGGTGGCAGC	14
Clones A26, A58, B1, B45	ATACCTACGGGTAGCAGC	
CFX109	ACGGCTGAGGAACACGTG	8
Clone A58	ACGGGTGAGTAACATGTT	
Clones A26 and B45	ACGGGTGAGTAACGCGTT	
Clone B1	ACGGGTGAGTAATGCGTT	
CFX784	GGGATTAGAGACCCCGGT	8
Clones A26, A58, B1, and B45	GGGATTAGAAACCCCGGT	

[a] Mismatches are underlined.

CFX197comp (Table 1) was used in combination with the CXF197 probe to reduce the likelihood of false positives with sequences containing a single known mismatch with its target site (sequence accession numbers ZA3635c and ZA3612c from uncultured bacterioplankton). Its effectiveness could not be assessed here. After validation against biomass from the Bendigo EBPR plant, formamide concentrations of 35 and 40% were selected for the CFX223 and CFX197 probes, respectively. Two helper probes CFX H202 and CFX H241, whose sequences are given in Table 1 were also designed for use with the CFX223 probe designed from the A58 clone sequence, which by necessity targets the relatively inaccessible region IV of the 16S rRNA of *Escherichia coli* (19). However, no obvious increase in fluorescence signal strength was noticed when these helper probes were used either individually or in combination with the CFX223 probe on biomass from the Bendigo plant.

When both targeted probes were applied to samples from Bendigo, filaments with the typical type 0092 morphotype (variant A) fluoresced with the CFX197 probe (Fig. 1b), while the CFX223 probe lit up the thinner (variant B) Neisser-positive *Chloroflexi* filaments (Fig. 1c). Whether these two variants represent different taxa of this filament morphotype is not clear from the phylogenetic data, and such a decision should be delayed until more sequence data become available. Again, FISH fluorescent signals were distinctively uneven and granu-

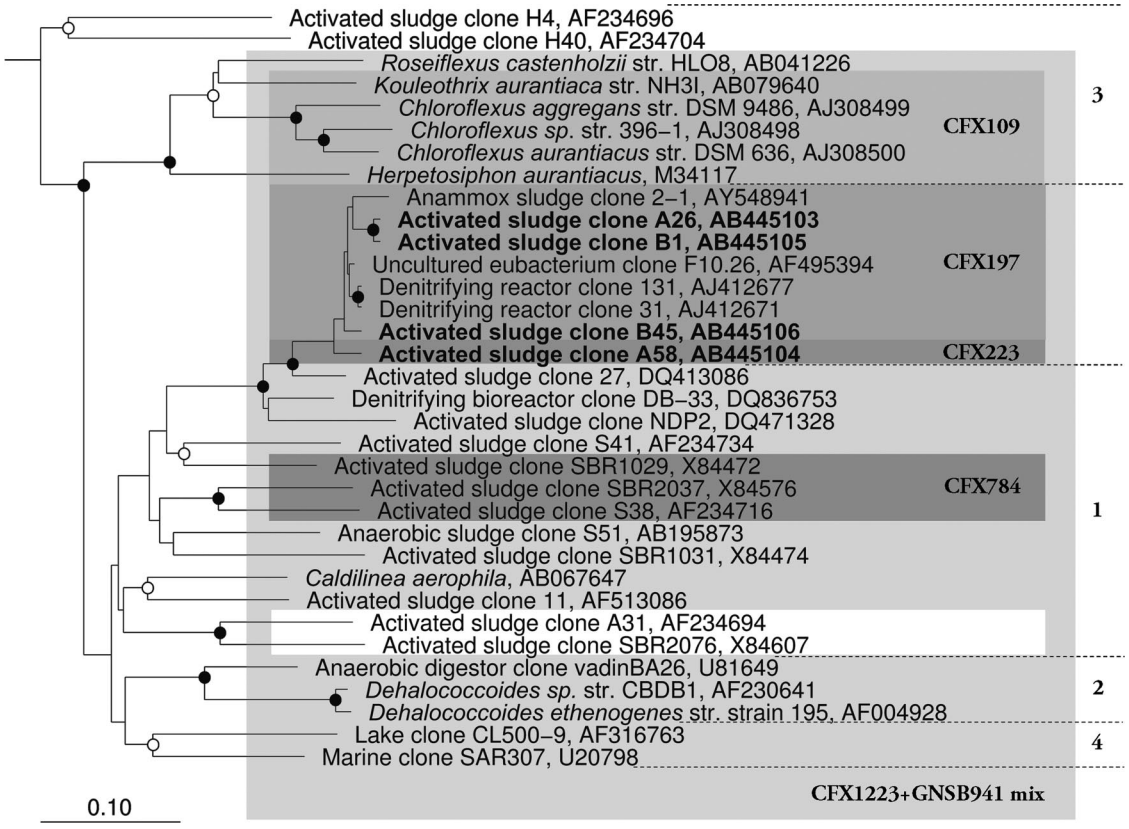

FIG. 2. Maximum-likelihood phylogenetic tree of the 16S rRNA gene sequences obtained in the present study and representatives from the *Chloroflexi* phylum. All sequences were at least 1,200 bp long except for AF495394, which was added in later using the quick-add function in ARB. Shading illustrates the coverage of each individual FISH probe. The scale bar corresponds to 0.1 substitutions per nucleotide position. Bootstrap values are calculated as a percentage of 1,000 analysis and are only indicated for values of ≥75%. Symbols: ○, bootstrap value of ≥75%; ●, bootstrap value of ≥95%.

lar. No other cells fluoresced with either probe that did not also fluoresce with the non-EUB probe.

polyP and PHA staining reactions. None of the type 0092 filaments stained positively for either polyP or PHA in any of the biomass samples examined.

FISH-based plant surveys. When biomass samples from full-scale EBPR and non-EBPR plants in eastern Australia were screened by FISH after Neisser staining for the presence of the type 0092 morphotype, most contained high levels of this filament. In all samples, type 0092 filaments fluoresced strongly with the CFX197 probe (Fig. 1b). Application of the CFX223 probe to the same samples revealed the presence of a thinner Neisser-positive type 0092 filament (Fig. 1c) in most of them (Table 2). They were less frequently seen in the non-EBPR biomasses and always at much lower abundances (Daylesford and Carrum).

DISCUSSION

This study has resolved the phylogeny of the Neisser-positive filament morphotype Eikelboom type 0092 commonly seen in Australian activated sludge plants is a member of the *Chloroflexi*. It also describes 16S rRNA targeted oligonucleotide sequences for unequivocal in situ identification by FISH of its two morphological variants, differing in their filament diameter. Application of these FISH probes to biomass samples from several Australian EBPR plants several thousand kilometers apart and with different operating configurations suggests that these two type 0092 variants each consist of a single phylotype, since they always responded to either the CFX197 or the CFX223 probes. However, all FISH-probed type 0092 cells had an unusual appearance, where the fluorescent signal from each was localized, a finding consistent with their ribosomes being in aggregates and not uniformly distributed within their cells (Fig. 1b and c). A similar arrangement has not been reported previously, but some *Planctomycetes* cells also show uneven FISH signal distribution, thought to arise from ribosome association with peripheral internal membranes (14). The Neisser negative filaments did not respond to any of the *Chloroflexi* targeted probes but instead fluoresced with the CFB719 probe designed against the *Bacteroidetes*. Whether these are the same filaments described by Lemmer et al. (30) or Kragelund et al. (26) was not examined here.

The successful eventual phylogenetic placement of type 0092 exploited preliminary FISH data, where screening by probing showed both were members of the *Chloroflexi*. The *Chloroflexi* 16S rRNA clones possessing or lacking these and other known probe signature target sequences could then be identified (Table 3). Thus, neither of the two filament morphotype variants identified here responded to any of the three EUBmix FISH probes designed to target members of the domain *Bacteria* (14), a feature shared by several other members of this phylum (8, 27). Such an outcome must impact on FISH quantification of *Chloroflexi* in natural communities if, as commonly used, it is based on calculating their biovolume percentages of EUBmix fluorescing cells (15). Redesigning the EUBmix probes to accommodate such populations is not straightforward, since the sequence diversity of the EUBmix probe target sites among them is substantial (T. Nittami, unpublished data; P. H. Nielsen, unpublished data). Several additional probes would be required to embrace this diversity.

The selected *Chloroflexi* sequences generated in the present study did not cluster closely with sequences in the preexisting divisions 1 and 3 of the *Chloroflexi* (8), thought to contain most of the activated sludge members of this phylum, but instead form a distinct adjacent grouping (Fig. 2). Thus, these type 0092 are not closely related to the other described *Chloroflexi* activated sludge filamentous bacteria Eikelboom type 1851 and "*Kouleothrix aurantiaca*" (6, 27) or the "*Nostocoida limicola*" morphotype of Schade et al. (44), whose 16S rRNA sequence is only ca. 80% similar. The FISH probes described here for type 0092 should now be applied more widely to samples from plants in other parts of the world. This will resolve the important question of whether members of this single filament morphotype are phylogenetically diverse, as the reports of Lemmer et al. (30) and Schade et al. (45) might suggest. A similar situation has been reported with several filament morphotypes including, for example, "*Nostocoida limicola*" II (31, 37, 44), type 021N (31, 49), and *Haliscomenobacter hydrossis* (26).

Several reports have suggested that the *Chloroflexi* and type 0092 are frequent members of EBPR communities (7, 8, 24), although whether they play any role in phosphate removal in these communities is uncertain. None in samples taken at the end of the aerobic stage stained positively for polyP in our study. Both variants occurred together in most of the EBPR plant samples examined, suggesting that they share an ecophysiology well suited to the alternative anaerobic:aerobic feast: famine conditions deliberately established in these processes (41, 46), although they were also seen in non-EBPR biomass samples (Table 2). Which features might be competitively advantageous to such populations would include an ability for anaerobic substrate assimilation for the synthesis of storage material such as poly β-hydroxyalkanoates. This may enable them to grow in the aerobic zone of EBPR plants in the absence of other exogenous metabolizable substrates (47) and consequently thrive in a highly competitive environment. However, Nile blue A staining failed to reveal the presence of PHA in these FISH-probed filaments in any biomass sample examined. Whether they possess a capacity for anaerobic substrate assimilation may be elucidated with FISH in combination with MAR (FISH/MAR). Where FISH/MAR was applied to other unidentified *Chloroflexi* in non-EBPR-activated sludge communities treating industrial wastes (27), the data suggested that they assimilated substrates actively only under aerobic conditions. This probably does not apply to all activated sludge populations, and certainly anaerobic *Chloroflexi* have been isolated from a range of environments (54). No mention was made (27) as to whether any of their activated sludge *Chloroflexi* synthesized PHA or polyP in situ. Clearly, more work is needed with FISH/MAR before their frequent appearance in EBPR plants can be explained, and their functional roles better understood. The FISH probes described here should make a valuable contribution to this important task.

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