Are some putative glycogen accumulating organisms (GAO) in anaerobic : aerobic activated sludge systems members of the \(\alpha\)-Proteobacteria?

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Activated sludge plants designed to remove phosphorus microbiologically often perform unreliably. One suggestion is that the polyphosphate-accumulating organisms (PAO) are out-competed for substrates by another group of bacteria, the glycogen-accumulating organisms (GAO) in the anaerobic zones of these processes. This study used fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) to analyse the communities from laboratory-scale anaerobic : aerobic sequencing batch reactors. Members of the genus *Sphingomonas* in the \(\alpha\)-Proteobacteria were present in large numbers in communities with poor phosphorus removal capacity where the biomass had a high glycogen content. Their ability to store poly-\(\beta\)-hydroxyalkanoates anaerobically, but not aerobically, and not accumulate polyphosphate aerobically is consistent with these organisms behaving as GAO there. No evidence was found to support an important role for the \(\gamma\)-Proteobacteria as possible GAO in these communities, although these bacterial populations have been considered in other studies to act as possible competitors for the PAO.

INTRODUCTION

Many activated sludge plants are designed to remove phosphorus microbiologically, by a process called enhanced biological phosphorus removal (EBPR) (Seviour *et al.*, 2003). All have one feature in common, in that the biomass is recycled repeatedly through anaerobic : aerobic zones, a strategy considered necessary to provide the polyphosphate-accumulating organisms (PAO) with a selective advantage (Seviour *et al.*, 2003). The PAO are thought to assimilate substrates present in the incoming sewage in the anaerobic zone and these are rapidly converted to intracellular storage compounds like poly-\(\beta\)-hydroxyalkanoates (PHA). Stored polyphosphate (polyP) is used as the energy source for PHA synthesis with the anaerobic release of orthophosphate (P). Then in the aerobic zone, where exogenous substrates are no longer available, the PAO can utilize the stored PHA as carbon and energy sources to grow and accumulate P as polyP. Glycogen synthesis is also thought to occur here to replenish the glycogen stores depleted under anaerobic conditions in supplying reducing power and probably energy for PHA synthesis (Seviour *et al.*, 2003).

EBPR systems are notoriously unreliable (Seviour *et al.*, 2003) and their performance often deteriorates for no clear reason. Not all explanations for such events allow for any role of microbes in process failure (Schönborn *et al.*, 2001) and many that do usually lack any convincing supporting microbiological evidence (Filipe *et al.*, 2001; Whang & Park 2002). Cech & Hartman (1993) noticed large numbers of cocci in distinctive tetrads, a morphotype they called the ‘G-bacteria’, in a reactor showing poor EBPR capacity that was fed glucose. These cocci have since been called tetrad-forming organisms or TFO (Tsai & Liu 2002). The original proposal, which has received support (reviewed by Seviour *et al.*, 2000, 2003) was that these TFO were out-competing the PAO by assimilating substrates in the anaerobic zone, utilizing them for PHA production, but with no concomitant P release. This stored PHA supported their growth in the aerobic zone, but now with glycogen accumulation instead of polyP storage. Consequently, these populations have been referred to as the glycogen-accumulating organisms (GAO) (Liu *et al.*, 1997; Bond *et al.*, 1999; Crocetti *et al.*, 2002).

However, the precise identity of these GAO is still largely unknown. Whether some of the TFO morphotype, now known from cultured isolates to be phylogenetically diverse (Seviour *et al.*, 2000), are GAO is also unclear. Isolates of
TFO from a reactor showing poor EBPR capacity by Cech & Hartman (1993) were identified as members of an α-proteobacterial genus, *Amaricoccus* (Maszenan et al., 1997), which in pure culture failed to synthesize polyP aerobically. While they could synthesize glycogen aerobically, they did not assimilate either acetate or glucose anaerobically (Falvo et al., 2001), and PHA synthesis occurred aerobically but not anaerobically. These are not physiological properties expected of a GAO (Hesselmann et al., 1999; Crocetti et al., 2002). However, it is possible that *Amaricoccus* spp. were not the organisms directly responsible for the poor EBPR seen by Cech & Hartman (1993).

Large coccobacilli have often been seen in reactors with low EBPR capacity, and from fluorescence in situ hybridization (FISH) these appeared to be β-Proteobacteria (Bond et al., 1999). However, considerable molecular data (Hesselmann et al., 1999; Nielsen et al., 1999; Liu et al., 2000; Dabert et al., 2001; Crocetti et al., 2002; Kong et al., 2002b) suggest that these coccobacilli are all phylogenetically closely related members of the γ-Proteobacteria. This conflict has since been shown to arise from the original design of the probes and their target sites, which differ only by a single nucleotide for these two subdivisions of the Proteobacteria (Yeates et al., 2003). Crocetti et al. (2002) and Levantesi et al. (2002) both considered that these γ-proteobacterial coccobacilli were GAO, a conclusion based not on any direct demonstration of *in situ* glycogen storage in these populations, but on their abilities to store PHA anaerobically and not aerobically, and inability to store polyP either anaerobically or aerobically (Hesselmann et al., 1999), as detected by staining after FISH probing. They called these ‘*Candidatus Competibacter phosphatis*’, implying that these were the populations which were competing with the PAO, and hence those responsible for EBPR deterioration (Crocetti et al., 2002).

However, these γ-Proteobacteria are sometimes found in communities with high EBPR capacity too (Liu et al., 2000) and do not always represent major populations in systems with low EBPR capacity and biomass with high glycogen contents (Kong et al., 2001, 2002a). Furthermore, Levantesi et al. (2002) and Crocetti et al. (2002) detected large numbers of γ-proteobacterial TFO in an EBPR community. They were not *Amaricoccus* spp. by FISH probing, but their identity and possible role as GAO were not pursued further, even though some, like the putative γ-Proteobacteria GAO discussed above, stained for PHA when grown under anaerobic but not aerobic conditions.

Earlier, Kong et al. (2001) detailed the structure–function relationships in several sequencing batch reactor (SBR) communities, showing that their community with no EBPR capacity was dominated by large z-proteobacterial TFO, as well as Gram-positive bacteria. Among these, *Micropruina glycogenica* (Shintani et al., 2000) was held responsible for the high glycogen content of the biomass (Kong et al., 2001). In another SBR biomass with very low EBPR capacity and high glycogen content (Kong et al., 2002a), FISH data showed the community was again dominated by z-proteobacterial TFO. Very few β-Proteobacteria or Gram-positive bacteria, which dominated communities with high EBPR capacity, were seen. However, the z-proteobacterial populations could not be identified, and neither denaturing gradient gel electrophoresis (DGGE) profiles nor clone libraries constructed from these biomasses using universal 16S rRNA gene PCR primers revealed z-Proteobacteria in proportions commensurate with the FISH data (Kong et al., 2001, 2002a). The possibility of bias in the DNA extraction from these communities was discussed as a possible reason for this (Kong et al., 2001).

This paper provides *in situ* evidence to support the view that the putative GAO in these communities are previously undescribed members of the genus *Sphingomonas* in the γ-Proteobacteria. It also suggests that the putative γ-proteobacterial GAO reported elsewhere (Crocetti et al., 2002; Kong et al., 2002b) are not significant there.

**METHODS**

**Microbial communities in SBR systems used in this study.** Operational conditions for the SBR systems used here for community analyses have been detailed previously. They include the non-P removing (SBR6) system fed acetate/glucose (Kong et al., 2001) and the three SBR systems fed acetate at differing carbon/phosphorus ratios. Each of these three showed different EBPR capacities and have been described in detail by Kong et al. (2002a). They are referred to here as SBR7, SBR8 and SBR9, and their main operational features and the chemical properties of their respective ‘steady-state’ biomasses are given in Table 1.

**Clone library construction.** Genomic DNA was extracted from biomass samples from these SBRs with the methods detailed previously (Kong et al., 2001). Preferential PCR amplification of the 16S rRNA genes from the z-Proteobacteria was attempted using as primers the ALF1b 16S rRNA-targeted oligonucleotide probe sequence (Manz et al., 1992) (5’-CTGGCTCAGAYCGAACG-3’) as the forward primer and the bacterial 16S rRNA gene 907r reverse primer (5’-CCGTCAATTCTTGGTGTTT-3’). The specificity of the ALF1b oligonucleotide probe sequence for the z-Proteobacteria is known to be low (www.microbial-ecology.de/probebase; Loy et al., 2003) and members of other bacterial groups were detected in the clone libraries obtained (see later). The PCR protocol used was that described by Felske et al. (1997), with an annealing temperature of 52 °C. DMSO (3 μl of a 50% solution) was added to each reaction to assist in amplification. PCR products were purified with the Concert Rapid PCR Purification System (Life Technologies) and cloned into the p-GEM Easy T Vector System II (Promega) according to the manufacturer’s instructions. This approach allowed construction of an z-proteobacterial clone library for each SBR community. Plasmids from each clone library were extracted with the QiAprep Spin Miniprep Kit (Qiagen) and the DNA size of the inserts was confirmed (approx. 950 bp) after their digestion with EcoRI. Sequencing of the 16S rRNA gene fragments was carried out as described previously (Kong et al., 2001). Sequences were aligned with CLUSTALW (accurate), from which maximum-likelihood relationships were estimated with DNAML and bootstrapped from 1000 trees with SEQBOOT, programs (Felsenstein, 1989) which are available on BioManager through ANGIS (www.angis.org.au). Possible chimera formation was checked with the ChimeraCheck program available on the Ribosomal Database Project II (Cole et al., 2003) and all those detected (a total of 3) were excluded from the study.
biosomes. DGGE analyses of the cloned fragments were used to compare their migratory positions with the bands generated from the SBR community genomic DNA templates separated by DGGE, adopting basically the same approach as Kong et al. (2001). This method was chosen in preference to the more common procedure of excising individual DGGE bands for sequencing, which is known to be problematic (Heuer et al., 2001). Only those cloned fragments which had identical migratory mobilities with the bands detected in DGGE profiles of genomic DNA from each community were then sequenced. A 30–70% gradient gel (8% acrylamide) was chosen for these separations because, of the several screened, this gave the best resolution and sharpest bands with the large fragments (approx. 950 bp) generated from PCR with the ALF1b and 907r primers. This method of screening the clones was preferred over the more usual RFLP fingerprinting method (Wagner & Loy, 2002), which does not permit such a direct elucidation of these relationships. Partial 16S rRNA gene sequences (approx. 950 bp) were obtained from most of the DNA cloned fragments with migratory properties corresponding to those genomic DNA fragments of interest from each SBR, allowing their tentative identification. No evidence was seen to suggest that any of these large fragments remained undenatured and moved towards the bottom of the gels.

**FISH probe design.** The clone sequence data from identified separated DGGE DNA fragments from the SBR communities were then used to design 16S rRNA targeted oligonucleotide probes against populations of the *z-Proteobacteria* detected in each, with programs available through BioManager (ANGIS). Candidate probe sequences were generated with the software package Prime (Genetics Computer Group, GCG) and the likely specificity of each was then assessed with BLASTN (Altschul et al., 1997). The probe sequences for the *z-Proteobacteria* described here are given in Table 2. As it was not possible to validate these probes against pure cultures of the *z-Proteobacteria* of interest, biomass samples corresponding to the SBR source of each clone were used instead. The strength of fluorescent signal and specificity of each of the probes, as judged from the morphology of the cells fluorescing with each, was compared over a range of formamide concentrations (from 0 to 40% in 5% steps) and the optimum formamide concentration was then selected (Weller et al., 2000; Rocetti et al., 2002). It is understood that this approach may mean that other unsequenced bacteria in different biomass samples may respond positively to the probes described

### Table 1. Summary of the chemical properties of SBR bio-masses during the anaerobic phase (Kong et al., 2001, 2002a)

<table>
<thead>
<tr>
<th>Variable</th>
<th>SBR6 (%)</th>
<th>SBR7 (%)</th>
<th>SBR8 (%)</th>
<th>SBR9 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate load</td>
<td>64-1</td>
<td>34-9</td>
<td>34-8</td>
<td>41-3</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate utilization</td>
<td>7-3</td>
<td>32-2</td>
<td>32-1</td>
<td>33-0</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1}) h(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose load</td>
<td>23-3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose utilization</td>
<td>112-2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1}) h(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycogen consumption</td>
<td>5-5</td>
<td>13-5</td>
<td>27-8</td>
<td>25-9</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA production</td>
<td>21-7</td>
<td>71-5</td>
<td>55-8</td>
<td>52-1</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/C ratio</td>
<td>NA</td>
<td>1:10</td>
<td>1:20</td>
<td>1:50</td>
</tr>
<tr>
<td>P released [mg C (g vsS)(^{-1})]</td>
<td>ND</td>
<td>27-0</td>
<td>21-4</td>
<td>2-4</td>
</tr>
<tr>
<td>P release rate</td>
<td>ND</td>
<td>27-0</td>
<td>21-4</td>
<td>1-6</td>
</tr>
<tr>
<td>[mg C (g vsS)(^{-1}) h(^{-1})]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomass P content (%)</td>
<td>ND</td>
<td>8-6</td>
<td>6-4</td>
<td>2-5</td>
</tr>
<tr>
<td>(%, w/w)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomass glycogen levels</td>
<td>24</td>
<td>7-5</td>
<td>12-5</td>
<td>16-9</td>
</tr>
<tr>
<td>(%, w/w)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

NA, Not applicable; ND, not detected; vsS, volatile suspended solids. *Values detected at the end of the aerobic phase of each run.

### Community fingerprinting with DGGE profiling.
Plasmids extracted from host cells of the clone libraries and the genomic DNA originating from each SBR community were both analysed by DGGE profiling, following the experimental approach detailed by Kong et al. (2001). The aim was to preferentially amplify the populations of the *z-Proteobacteria* which might be present in the SBR

### Table 2. 16S rRNA targeted oligonucleotide probes used in this study

Both standard probe names and trivial name abbreviations are given for each probe sequence. Stringency is given as the concentration of formamide used for hybridization of probes for FISH during this study and determined for the probes designed in this study as detailed in the text.

<table>
<thead>
<tr>
<th>Standard probe name</th>
<th>Abbreviation</th>
<th>Sequence (5’-3’*)</th>
<th>Target</th>
<th>Stringency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-D-s91a-0578-b-A-20</td>
<td>SBR9-1a</td>
<td>AAGCGCAAGTTCCCAAGGTG</td>
<td>SBR9 clone 1 (AY254694)</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>S-D-s91b-0778-b-A-18</td>
<td>SBR9-1b</td>
<td>TGTATAGGCGCTTAGACCT</td>
<td>SBR9 clone 1 (AY254694)</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>S-D-s8_4-0752-a-A-20</td>
<td>SBR9-4</td>
<td>CACCGAAGCATAAGTGCCC</td>
<td>SBR9 clone 4 (AY254692)</td>
<td>20</td>
<td>This study</td>
</tr>
</tbody>
</table>

*The three bacterial probes were applied as a mixture, EUBmix.
here, especially since these probes were designed against only partial sequences of individual clones (i.e. only 950 bp). All probes were supplied commercially by ProOligo and labelled with either the Cy3 or Fluos fluorochrome.

**FISH analyses of SBR communities.** Biomass samples from each SBR were fixed in 4 % paraformaldehyde and FISH was carried out with the probes listed in Table 2, essentially by the methods of Aman & Doi (1996). Cells were permeabilized for the probes designed in this study, as described previously (Kong et al., 2001). VectaShield (Vector Laboratories) was used to mount hybridized samples, which were then examined with a Nikon Eclipse 800 epifluorescence microscope. For each biomass sample, at least 10 frames, with an estimated total of approximately 2000 bacteria in these fields of view, were captured at random. The total area of specific bacterial populations responding to individual Cy3-labelled FISH probes was measured using Scion Image beta 4.0.2 Windows version (Scion) and expressed as the percentage of the total cell area (Bouchez et al., 2000) fluorescing with the EUBmix probe of Daims et al. (1999). This semi-quantitative epifluorescence technique does not accommodate readily the problems arising from the three-dimensional nature of activated sludge flocs. However, the TFO cells of particular interest in this study were usually arranged in freely dispersed two-dimensional sheets where these problems were less apparent. Even so, the differences between replicate determinations were often marked and so standard deviations for the values are given in the Results.

**Staining of PHA and polyP in SBR biomasses.** Bacterial cells accumulating PHA in each SBR biomass were visualized with the staining protocol of Kitamura & Doi (1996). Briefly, fixed biomass samples from the end of the anaerobic phase were treated with nile blue A (100 mg l−1) in absolute ethanol for 30 min. After microscopic examination, de-staining was carried out by immersion of slides in absolute ethanol for 30 min. PolyP accumulation in samples from the aerobic stages was detected with 4',6-diamidino-2-phenylindole (DAPI) with the method of Liu et al. (2001). FISH analysis was carried out as previously described in this paper to identify the PHA- and polyP-accumulating populations.

**RESULTS**

**DGGE profiles of 16S rRNA gene fragments from SBR communities and identification of individual fragments**

For each SBR community, 30 cloned fragments were screened by DGGE. Those fragments co-migrating with the fragments derived from the corresponding community genomic DNA were then sequenced. Although most of these were α-Proteobacteria (60 %), some were β-Proteobacteria (33 %), γ-Proteobacteria (3 %) and Actinobacteria (3 %).

DGGE profiles of the ALF1-907r-generated DNA fragments from the communities in SBR6, 7, 8 and 9 are shown in Fig. 1. As expected from previous FISH and chemical analyses (Kong et al., 2001, 2002a), profiles obtained for SBR communities 7 and 8, both with very similar and high EBPR performances, are almost identical to each other, and substantially different to those from SBR communities 6 and 9, with little or no EBPR capacity. Most of the α-proteobacterial clone fragments in the SBR7 community profile were also seen in the community profile in SBR8. The SBR9-1 clone fragment was of particular interest. Although present in all communities profiled, it had a much greater fluorescent intensity on DGGE gels generated from the communities in SBR6 and 9, and especially the latter (Fig. 1). This sequenced clone was only 92 % similar to its closest relative, an uncultured *Sphingomonas* sp. (AF181572). About 30 % of the clones screened from these two SBRs had identical DGGE migratory mobilities to the SBR9-1 clone.

A tree showing the phylogenetic diversity among the DGGE-resolved fragments of α-proteobacterial DNA from biomasses from these SBRs, successfully identified from sequenced cloned fragments with identical DGGE migratory behaviours, is given in Fig. 2. The only strongly fluorescent band not identified from DGGE analysis of the clone library was band X seen in the SBR6 DGGE profile (Fig. 1). Repeated attempts to sequence it after its excision from the gel were unsuccessful because of contamination with other DNA. The tree reveals that the phylogenetic diversity among the α-Proteobacteria detected by DGGE in the four SBR communities was quite limited and all the populations detected were most closely related to members of the genus *Sphingomonas* in subgroup 4 of this subdivision (Fig. 2). Although DGGE theoretically separates same-sized fragments of DNA differing in only a single base (Myers et al., 1985), some fragments with identical migratory mobilities (e.g. SBR clones 6-8 and 6-9), gave sequences that were quite different to each other (Figs 1 and 2). The clone sequences that were obtained from populations not in the α-Proteobacteria are omitted from this tree.
Semi-quantitative analysis of populations in SBR communities with FISH probing

When fixed biomass samples from each of the SBRs were probed with both the FISH probes SBR9-1a and SBR9-1b designed against the SBR9-1 clone (Table 2), cells responding to these probes emerged as the dominant population (71 ± 15 % of total cell area) in the SBR9 community, where a very low EBPR capacity accompanied high biomass glycogen levels. This population was also numerically significant in the SBR6 community (19 ± 15 % of total cell area) with no EBPR, but a high glycogen content, and barely detectable (< 1 % of total cell area) in SBR7 and 8 communities, both with high EBPR performance and relatively low glycogen contents (Kong et al., 2002a). These probe sequences have been deposited in the probeBase.net database (www.microbial-ecology.de/probebase/; Loy et al., 2003). FISH probing revealed that the cells responding to the SBR9-1a and SBR9-1b probes also fluoresced with the ALF968 probe (Neef et al., 1999). They were cocci (1·2–1·5 μm diam.) arranged in regular tetrads, i.e. with a typical TFO morphotype, often appearing in large clusters (Fig. 3a, b). This was the α-proteobacterial morphotype shown previously in SBR9 (Kong et al., 2002a) by microscopy to dominate this community. Double probing with Cy3-labelled SBR9-1a and Fluos-labelled SBR9-1b probes (Fig. 3c) clearly show that these probes target the same population, since the TFO clusters appear generally as yellow. Of the two probes designed to target this clone (Table 2), the SBR9-1a probe always gave the brighter signal regardless of the fluorescent tag and this is probably the reason why some of the TFO in Fig. 3(c) appear slightly redder than yellow. It has at least three mismatches with all other 16S rRNA gene sequences in GenBank, while the SBR9-1b has at least two mismatches.

Although oligonucleotide probes were designed against other clone sequences, with one exception they are not discussed further here. They were considered to be unsuitable for studies of this kind because of their low probe specificity or they hybridized with higher numbers of cells in the biomass samples with low glycogen contents (i.e. SBR7 and SBR8). As the FISH probe SBR9-1a has an insert at position 7 not found in the target site of clone SBR8-4 and the FISH probe SBR9-1b has two mismatches with the clone SBR8-4 target site, neither probe would detect cells with this sequence. Furthermore, the cells responding to the clone SBR8-4 FISH probe were not the distinctive TFO, but small irregularly clustered cocci and rods (data not shown). For reasons not understood, none of the α-proteobacterial TFO in these SBRs responded positively to the 16S rRNA-targeted probe for Sphingomonas spp. (Table 2) described by Neef et al. (1999), even though all the sequenced α-proteobacterial clones possessed the target sequence for this probe. None of
Fig. 3. FISH of biomass samples using the 16S rRNA-targeted probes SBR9-1a and SBR9-1b designed against the \( \alpha \)-proteobacterial SBR9-1 clone in SBR communities. (a) SBR9 biomass seen under phase-contrast. (b) The same cells hybridized with the SBR9-1a clone FISH probe showing fluorescing TFO. (c) SBR biomass double-probed with the Cy3-labelled SBR9-1a clone (red) and Fluos-labelled SBR9-1b clone (green) FISH probes. TFO appear mainly as yellow-fluorescing cells showing that they are targeted by both these probes. (d) SBR9 anaerobic reactor biomass hybridized with the SBR9-1a clone probe (left frame) and corresponding cells after nile blue A staining for PHA (right frame), showing that the TFO contain PHA. (e) SBR9 aerobic reactor biomass hybridized with the SBR9-1a clone probe (left frame) and corresponding cells after nile blue A staining for PHA (right frame), showing that the TFO contain no PHA. (f) Cells in tetrads in biomass from the full-scale Bendigo EBPR plant after hybridization with the SBR9-1a clone FISH probe. Bars, 10 \( \mu \)m.
the many attempts made to isolate and grow the TFO present in these SBRs with the methods described by Maszenan et al. (1997) for TFO and Sphingomonas spp. (Neef et al., 1999) were successful.

**PHA and polyP production by the α-Proteobacterial TFO**

Sequential nile blue A staining and FISH showed that cells fluorescing with the SBR9-1a probe in biomass samples taken at the end of the anaerobic period from SBR6 and 9 contained PHA (Fig. 3d), while those taken under aerobic conditions contained neither PHA (Fig. 3e) nor polyP (data not shown).

**FISH probing of putative γ-proteobacterial GAO in SBR communities**

When the FISH probes previously designed against the putative γ-proteobacterial GAO were applied to these SBR communities, barely detectable numbers of cells (<4% of total cell area) fluoresced with any of the γ-proteobacterial GAO probes (Table 2) of Crocetti et al. (2002) and Kong et al. (2002b) in any of the communities. Those few cells that did were large (>1·5 μm diam.) coccobacilli, often in pairs or loose clusters, consistent with their reported appearance in other activated sludge biomass samples (Crocetti et al., 2000, 2002; Kong et al., 2002b). These data suggest that these putative γ-proteobacterial GAO were minor populations there and so were probably not mainly responsible for the marked levels of glycogen synthesis detected, especially in the SBR6 and 9 communities.

**Occurrence of putative GAO in full-scale EBPR plants**

When the FISH SBR9-1a probe was applied to biomass samples taken from 10 full-scale EBPR plants from eastern states of Australia, very few contained this population, as might be expected of the communities in plants with high EBPR capacity, as these were (M. Beer, unpublished). The one exception was the Bendigo plant, Victoria, where >5±3% of the total cell area responding to the EUBmix probe fluoresced with it. Again the cells appeared as typical TFO but scattered throughout the flocs (Fig. 3f) and none stained positively for polyP with DAPI (Liu et al., 2001). This plant often shows erratic EBPR performance, although its biomass glycogen levels were not estimated here. Only small numbers of cells (<1% of total cell area) responding to the γ-proteobacterial GAO probes were detected by FISH in biomasses from these plants, as might be expected, and in most none were seen (data not shown).

**DISCUSSION**

There now seems little doubt that a physiological group of bacteria capable of accumulating intracellular glycogen exists in activated sludge systems, but their identity and possible role in the population dynamics of EBPR systems are still unclear. Glycogen is not as readily detected directly by staining as PHA and polyP in bacterial cells (Serafim et al., 2002). So the GAO have been defined less strictly in physiological terms as those bacterial populations unable to store polyP aerobically and capable of storing PHA anaerobically but not aerobically (Hesselmann et al., 1999; Crocetti et al., 2002; Levantesi et al., 2002). These are the attributes expected in organisms that provide them with a potentially competitive edge against the PAO under anaerobic conditions (Hesselmann et al., 1999).

Most attempts, including ours, to grow any hypothesized GAO have been unsuccessful (Kong et al., 2001; Crocetti et al., 2002). When the FISH probes previously designed against the γ-Proteobacteria of Crocetti et al. (2002) and Kong et al. (2002b) pertain to the glycogen content (Kong et al., 2001, 2002b; Crocetti et al., 2002). Subsequent culture-independent 16S rRNA-based methods have then been used to try to relate the identity of these dominating morphotypes to their abilities to synthesize PHA and polyP anaerobically and aerobically.

This is essentially the method adopted in this study. The in situ physiological evidence presented here is as convincing as that from other similar studies, suggesting that the probable main candidates for GAO in the SBR9 community at least are likely to be members of the α-Proteobacteria, most closely related to Sphingomonas spp. (i.e. clone SBR9-1). These are phylogenetically different to the α-proteobacterial Amacricoccus spp. once considered as possible GAO candidates (Seviour et al., 2000). Sphingomonas spp. can be abundant populations in activated sludge communities and cells appear to form large aggregates within the flocs. However, tetrad cell arrangements have not yet been reported in activated sludge for members of this genus (Neef et al., 1999; Snaidr et al., 1997).

No convincing evidence was found to support an important role here in glycogen accumulation for the γ-Proteobacteria proposed by Crocetti et al. (2002) as putative GAO in their communities. Although the percentage of the total cell area of γ-Proteobacteria increased markedly to approximately 8% in the SBR9 biomass with a glycogen content of about 17% (w/w) dry weight, very few cells were detected by FISH in this study with the γ-proteobacterial GAO probes of either Crocetti et al. (2002) or Kong et al. (2002b).

In summary, the TFO responding to the SBR9-1a and SBR9-1b probes seems a highly likely candidate as a GAO, dominating the SBR9 biomass characterized by a high glycogen and low P content (Kong et al., 2002a). These cells were also numerically significant in the SBR6 community where M. glycogenica had been suggested previously as the major possible GAO (Kong et al., 2001).
It seems unlikely that all the GAO are representatives of a single population and, like the PAO now appear to be, are probably phylogenetically very diverse (Crocetti et al., 2002; Eschenhagen et al., 2003). The evidence presented here supports this. The availability of the FISH probe sequences for the SBR9-1 clone should now provide the opportunity to determine how widespread these \( \chi \)-Proteobacteria are, especially in full-scale plants where this study has shown they may exist, and whether, for example, they are the same or similar populations to the \( \chi \)-Proteobacteria seen in high numbers by Crocetti et al. (2002) in their community. However, it may be that these GAO do not always store glycogen under all conditions, which now seems to be the case with polyP and the Rhodococcus-related PAO (Wagner & Loy, 2002; Zilles et al., 2002). If so, this will further complicate attempts to relate numerical dominance of particular morphotypes to their functional roles in biomasses displaying deteriorated EBPR.

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REFERENCES


