Putative glycogen-accumulating organisms belonging to the *Alphaproteobacteria* identified through rRNA-based stable isotope probing

Rikke Louise Meyer,[†] Aaron Marc Saunders[†] and Linda Louise Blackall

Advanced Wastewater Management Centre, The University of Queensland, St Lucia, QLD 4072, Australia

Correspondence

Linda Louise Blackall
blackall@awmc.uq.edu.au

Deterioration of enhanced biological phosphorus removal (EBPR) has been linked to the proliferation of glycogen-accumulating organisms (GAOs), but few organisms possessing the GAO metabolic phenotype have been identified. An unidentified GAO was highly enriched in a laboratory-scale bioreactor and attempts to identify this organism using conventional 16S rRNA gene cloning had failed. Therefore, rRNA-based stable isotope probing followed by full-cycle rRNA analysis was used to specifically identify the putative GAOs based on their characteristic metabolic phenotype. The study obtained sequences from a group of *Alphaproteobacteria* not previously shown to possess the GAO phenotype, but 90 % identical by 16S rRNA gene analysis to a phylogenetic clade containing cloned sequences from putative GAOs and the isolate *Defluvicoccus vanus*. Fluorescence *in situ* hybridization (FISH) probes (DF988 and DF1020) were designed to target the new group and post-FISH chemical staining demonstrated anaerobic–aerobic cycling of polyhydroxyalkanoates, as per the GAO phenotype. The successful use of probes DF988 and DF1020 required the use of unlabelled helper probes which increased probe signal intensity up to 6.6-fold, thus highlighting the utility of helper probes in FISH. The new group constituted 33 % of all *Bacteria* in the lab-scale bioreactor from which they were identified and were also abundant (51 and 55 % of *Bacteria*) in two other similar bioreactors in which phosphorus removal had deteriorated. Unlike the previously identified *Defluvicoccus*-related organisms, the group identified in this study were also found in two full-scale treatment plants performing EBPR, suggesting that this group may be industrially relevant.

Received 14 August 2005

Revised 25 October 2005

Accepted 1 November 2005

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is a microbial process widely used for removing phosphorus (P) from wastewater (Mino *et al.*, 1998) and presents an environmentally friendly alternative to P removal by chemical precipitation. EBPR is facilitated by polyphosphate-accumulating organisms (PAOs) that take up P in excess of that required for normal cellular growth. When these microorganisms are exposed to alternating anaerobic and aerobic conditions where carbon is available only under anaerobic conditions, they take up volatile fatty acids (VFA) in the anaerobic period without the use of an external electron acceptor and use the VFA to synthesize polyhydroxyalkanoates

(PHAs) (Mino *et al.*, 1998; Seviour *et al.*, 2003). The energy and electrons needed for the uptake and transformation of VFA under anaerobic conditions come from intracellular glycogen and polyphosphate, and orthophosphate is released by the cells (Seviour *et al.*, 2003). In the following aerobic period, part of the intracellular PHA is converted to glycogen and part is oxidized, producing CO₂ and providing energy for growth and uptake of orthophosphate to replenish intracellular polyphosphate (Seviour *et al.*, 2003). The uptake of P in the aerobic period is greater than the release of P in the anaerobic period and a net removal of P from the bulk liquid therefore occurs by the end of the process cycle. At the end of the aerobic period, a fraction of the biomass is removed for a net removal of P from the system.

Glycogen-accumulating organisms (GAOs) are considered competitors to PAOs, as they can perform similar carbon transformations but do not take up P in excess of the requirement for growth (Liu *et al.*, 1996; Satoh *et al.*, 1992). GAOs possess the ability to take up VFA under anaerobic conditions, convert them to PHA, which is stored until the following aerobic period and then oxidized to CO₂ or

[†]Present address: Department of Microbial Ecology, Aarhus University, Ny Munkegade, 8000 Aarhus C, Denmark.

Abbreviations: COD, chemical oxygen demand; EBPR, enhanced biological phosphorus removal; FISH, fluorescence *in situ* hybridization; GAO, glycogen-accumulating organism; OTU, operational taxonomic units; PAO, polyphosphate-accumulating organism; PHA, polyhydroxyalkanoate; SBR, sequencing batch reactor; SIP, stable isotope probing; TFO, tetrad-forming organism; VFA, volatile fatty acids.

transformed to glycogen. The glycogen produced during the aerobic period provides energy and reducing equivalents for the carbon uptake and transformations that occur during the anaerobic period (Filipe *et al.*, 2001; Zeng *et al.*, 2003b). GAOs therefore compete with PAOs for VFA, and GAOs are known to be abundant in systems being operated for EBPR, but where P-removal is poor (Liu *et al.*, 1996; Oehmen *et al.*, 2004).

Only a few GAOs have been identified and as result of this, the role of different GAOs in deterioration events of the EBPR process has not been fully characterized. A deep-branching group of *Gammaproteobacteria* was identified as containing putative GAOs (Crocetti *et al.*, 2002; Kong *et al.*, 2002a; Nielsen *et al.*, 1999) and this group is known as the GB lineage (Kong *et al.*, 2002a) or by the name 'Candidatus Competibacter phosphatis' (Crocetti *et al.*, 2002). Using fluorescence *in situ* hybridization (FISH) probes targeting this group, Saunders *et al.* (2003) were able to show a negative correlation between the abundance of 'Candidatus Competibacter phosphatis' and the ratio of anaerobic VFA consumption to anaerobic P release in a number of full-scale wastewater treatment plants performing EBPR. This suggests that members of 'Candidatus Competibacter phosphatis' are probably GAOs playing an important role in the competition for VFA between PAOs and GAOs in EBPR in full-scale treatment plants.

Other bacteria, described as 'G-bacteria' (Cech & Hartman, 1993) or 'tetrad-forming organisms' (TFOs) (Levantesi *et al.*, 2002; Tsai & Liu, 2002), are putative GAOs in EBPR systems. Organisms of this morphotype have been linked to the deterioration of EBPR in lab-scale systems (Kong *et al.*, 2002b; Oehmen *et al.*, 2005c). Numerous isolates of TFOs have been obtained, but none of these organisms has been shown to display the GAO phenotype (Seviour *et al.*, 2003). Using culture-independent methods, two TFOs in the *Alphaproteobacteria* were recently identified from anaerobic- aerobic activated sludge biomass and shown to possess aspects of the GAO phenotype. One group is in the *Sphingomonadales* family (Beer *et al.*, 2004) and the other is related to the isolate *Deffluvicoccus vanus* (Wong *et al.*, 2004). While present in lab-scale systems, these organisms have been found in only a few full-scale EBPR plants and more knowledge of the diversity of GAOs that have the TFO morphotype is required.

In the study presented here, a directed approach using rRNA-based stable isotope probing (SIP) to target cells capable of anaerobic propionate uptake identified a distinct group of *Alphaproteobacteria* related to *D. vanus*, which displayed the PHA transformation component of the GAO phenotype. This study thereby adds to the diversity of known putative GAOs that may play a role in deterioration of EBPR.

METHODS

Reactor operation. Biomass from three lab-scale sequencing batch reactors (SBRs) and two full-scale wastewater treatment plants were

used in this study. Lab-scale SBR performance was monitored by weekly cycle studies, in which VFA, PHA and glycogen were measured at regular intervals over the cycle. Analytical methods are described by Oehmen *et al.* (2005a).

Details about each system are provided below, but only biomass from the Reactor 1 was used for SIP.

Reactor 1 was an 8-litre SBR configured for enrichment of GAOs. It was operated with sequential anaerobic and aerobic periods, where the initial concentrations of carbon and P in the reactor were 132 mg propionate l⁻¹ [200 mg chemical oxygen demand (COD) l⁻¹] and 2 mg PO₄-P l⁻¹. Influent was added only in the first 5 min of the anaerobic period and propionate, which was the only carbon source, was fully consumed before the aerobic period. The reactor was operated in a 6 h cycle; 130 min anaerobic period, 160 min aerobic period (O₂ set-point = 3.0 ± 0.2 mg l⁻¹) and 70 min settle and decant. Further details of the SBR operation and influent composition are given in the description of reactor GAO-2 in Oehmen *et al.* (2005a).

Reactor 2 was an 8-litre SBR configured for enrichment of PAOs. It was operated with a similar cycle configuration and O₂ setpoint as Reactor 1. The influent composition was identical, except that P was added at an initial concentration of 13.3 mg PO₄-P l⁻¹. Influent was added only in the first 7 min of the anaerobic period and propionate was fully consumed before the aerobic period. The reactor was monitored as described for Reactor 1. A further description of this reactor is given in Oehmen *et al.* (2005b).

Reactor 3 was a 5-litre SBR configured for simultaneous nitrification, denitrification and phosphorus removal (Zeng *et al.*, 2003a). The SBR was operated in a 6 h cycle, including a 1 h anaerobic period, a 4 h aerobic period (O₂ set-point of 0.35–0.50 mg l⁻¹) followed by a 1 h settle and decant period. Influent containing propionate as the only carbon source was added only in the first 7 min of the anaerobic period, leading to a propionate concentration of approximately 99 mg propionate l⁻¹ (150 mg COD l⁻¹), which was fully consumed before the aerobic period.

Full-scale plant A was configured as a five-stage Bardenpho process (Tchobanoglous & Burton, 1991) treating 12 Ml wastewater per day. The influent contained 240–360 mg biochemical oxygen demand l⁻¹ and 4–21 mg PO₄-P l⁻¹; the effluent contained less than 0.5 mg PO₄-P l⁻¹ during the sampling period.

Full-scale plant B was an oxidation ditch with an initial anaerobic reactor, giving a 'three-stage Phoredox'-type process (Tchobanoglous & Burton, 1991), which treated 7.5 Ml wastewater per day. The influent contained approximately 550 mg COD l⁻¹ and 11 mg PO₄-P l⁻¹; the effluent contained approximately 3 mg PO₄-P l⁻¹ (de Haas, 2005).

SIP. Biomass (50 ml) was sampled from Reactor 1 at the end of the aerobic period and transferred to a 100 ml bottle for ¹³C labelling. Sequential anaerobic-aerobic conditions were facilitated by sparging with O₂-free N₂ gas for 3 h followed by air sparging for 2 h. Biomass (6 ml) was removed at the end of the aerobic period, but no settle or decant periods were included in the cycle. HEPES (3.0 g l⁻¹; BDH) was added to buffer the medium and the pH was adjusted to 7.0 at the end of each anaerobic and aerobic period. Nutrients were added at the beginning of the anaerobic period proportionally to the nutrient concentration in the influent to the main SBR. ¹³C-labelled propionate (1,2-¹³C sodium propionate; ICON Isotopes) was the only carbon source. At the end of each anaerobic period, a 2 ml sample was filtered (0.22 µm polyethersulfone; Millipore) for VFA analysis to confirm that no propionate was detectable when aeration commenced, thus restricting ¹³C-uptake to organisms capable of anaerobic propionate uptake. VFA analysis was

carried out by HPLC (Shimadzu) with an HPX-87H 300 × 7.8 mm Bio-Rad Aminex ion exclusion column. After eight cycles, the incubation was terminated and the remaining biomass was divided into 2 ml aliquots for nucleic acid extraction.

Nucleic acid extraction and measurement of ^{13}C -labelling. RNA was extracted and pooled from two aliquots of 2 ml biomass using the FastRNA blue kit (Qbiogene), following the manufacturer's instructions. RNA was further purified with an RNeasy spin column (Qiagen). DNA was extracted using the FastDNA SPIN kit (Qbiogene), following the manufacturer's instructions, and quantified by spectrophotometric analysis at 260 nm.

The degree of ^{13}C -labelling of the DNA and RNA was then measured on extracted nucleic acids from biomass sampled before and after incubation with ^{13}C -propionate. Labelling was assessed by determining the $^{13}\text{C}/^{12}\text{C}$ ratio through online combustion of a sample in a Fisons elemental analyser (NA-1500 NC) coupled to a Micromass IsoPrime continuous flow stable isotope ratio mass spectrometer (CF-IRMS). The CF-IRMS measures the $^{13}\text{C}/^{12}\text{C}$ ratio (‰) of the sample against a global standard, expressed as the $\delta^{13}\text{C}$ value. To obtain sufficient sample material for the analysis (> 10 µg-C was required), 0.36 µg-C DNA or RNA was mixed with 10 µg-C glucose, corresponding to an approximately 28-fold dilution of the sample carbon with glucose-carbon.

Isolation of ^{13}C -labelled RNA. RNA concentration and integrity were determined using a 2100 Bioanalyser (Agilent Technologies), following the manufacturer's instructions. RNA (500 ng) was mixed with gradient buffer (0.1 M Tris/HCl, pH 8; 0.1 M KCl; 1 mM EDTA) to obtain a total volume of 0.9 ml. Deionized formamide (0.175 ml) and 4.1 ml caesium tri-fluoroacetate (CsTFA; Amersham) were then added, resulting in a mean density of 1.794 g ml⁻¹. The mixture was placed into 5.1 ml heat-sealable centrifuge tubes (Beckman) and centrifuged at 49 000 r.p.m. (130 000 g) for 60 h in an Optima TFX ultracentrifuge (Beckman). Immediately after centrifugation, each tube was carefully fractionated by injecting sterile water into the top of the tube at 0.2 ml min⁻¹ and collecting 10 fractions (0.4 ml) from a hole pierced in the bottom. The density of each fraction was measured with a temperature-controlled RFM340 refractometer (Bellingham & Stanley) using CsTFA and MilliQ water as standards.

cDNA synthesis, cloning and sequencing. RNA was isolated from each fraction by 2-propanol precipitation (Sambrook & Russell, 2001), then cDNA was synthesized using the Superscript III kit (Invitrogen) and the 27f primer (5'-AGAGTTTGATCMTGG-CTCAG-3'; Lane, 1992), following the manufacturer's instructions. 16S rRNA genes were then PCR-amplified from cDNA from each fraction using the 27f and 1492r (5'-TACGGYTACCTTGTTACG-ACTT-3'; Lane, 1992) primers. The size of each PCR product was confirmed by gel electrophoresis.

A clone library was prepared from the PCR product from the fraction with the highest density using the pGEM-T cloning vector (Promega) and OneShot competent *Escherichia coli* cells (Invitrogen). Cloned inserts were amplified using vector-specific primers (T7 and SP6) and screened using restriction fragment length polymorphism (RFLP) analysis with *MspI* (New England Biolabs). Clones from the most abundant RFLP patterns were selected for full-length 16S rRNA gene sequencing using BigDye Terminator 3.1 reaction chemistry and the following primers (Lane, 1992): 27f and 1492r (detailed above), and 530f (5'-GTGCCAGCMGCCGCGG-3') and 907r (5'-CCGTCAAATTCMTTTRAGTTT-3').

FISH probe design, optimization and evaluation. Sequence alignment and phylogenetic analysis of 16S rRNA gene sequences were performed using the software ARB (Ludwig *et al.*, 2004). BLAST (Altschul *et al.*, 1997) was used to identify closely related sequences

and these were also added to the database. Clone sequences were checked for chimeras by analysing fragments of each sequence in BLAST.

Phylogenetic trees were made using the maximum-likelihood algorithm (AxML) and bootstrap analysis was performed using maximum-parsimony with 1000 replicates. Sequences shorter than 1200 nt were added by the parsimony insertion tool after construction of the maximum-likelihood tree. These sequences are indicated by dashed lines. Probes were designed using the Probe Design function in ARB. Probe length was adjusted to obtain a T_m higher than 57 °C and helper probes were designed to have a melting temperature exceeding that of the labelled probes.

FISH was carried out according to the protocol of Amann (1995) and results visualized on a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM). FISH probes used in this study were EUBMIX for *Bacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999), DF1MIX (TFO_DF218 plus TFO_DF618) for a group of *D. vanus*-related *Alphaproteobacteria* (Wong *et al.*, 2004), SBR9-1a for *Sphingomonas*-related *Alphaproteobacteria* (Beer *et al.*, 2004), GAOMIX [probes GAOQ989, GAOQ431 (Crocetti *et al.*, 2002) and GB_G2 (Kong *et al.*, 2002a)] for '*Candidatus* Competibacter phosphatis', PAOMIX for '*Candidatus* Accumulibacter phosphatis' (probes PAO462, PAO651 and PAO846; Crocetti *et al.*, 2000) and ALF1b (Manz *et al.*, 1992) for *Alphaproteobacteria*.

FISH quantification was done by digital image analysis using Laserpix software (Bio-Rad). Samples were hybridized with the Cy3-labelled specific probe and Cy5-labelled EUBMIX (Amann *et al.*, 1990; Daims *et al.*, 1999) and 40 images were captured using the same CLSM settings. The abundance of cells targeted by the specific probe was calculated as the area of each image containing a positive signal for both the specific probe and the EUBMIX probes relative to the area only containing a positive signal for the EUBMIX probes. The value presented is a mean value for the 40 images.

FISH with the DF988 and DF1020 probes was optimized with respect to formamide concentration and the effect of helper probes by quantifying the FISH probe signal intensity. Signal intensity was determined for > 30 individual cells using image analysis with ImageJ software (<http://rsb.info.nih.gov/ij/>) on images captured using the same CLSM settings. The effect of helper probes was tested using 35 % formamide in the hybridization buffer and the effect of formamide in the range of 20–50 % was then tested at 5 % intervals while including the chosen unlabelled helper probes in the incubation. No pure cultures with less than 4 nt mismatches to either probe were available to be used as positive or negative controls. The probes therefore had to be optimized using the biomass from Reactor 1. This approach has been used previously (e.g. Erhart *et al.*, 1997).

Post-FISH chemical staining was carried out as detailed by Crocetti *et al.* (2000) and the staining of intracellular PHA was done according to the method of Ostle & Holt (1982).

RESULTS AND DISCUSSION

Conventional cloning

In our laboratory, TFOs belonging to the *Alphaproteobacteria* were abundant in the mixed microbial communities from three different anaerobic-aerobic lab-scale bioreactors that received propionate as the sole carbon source. Reactor 1 received a low influent PO₄-P concentration to enrich for GAOs. Reactors 2 and 3 received high

influent $\text{PO}_4\text{-P}$ concentrations to enrich for PAOs, but EBPR had deteriorated in both of the latter reactors. In Reactor 2, the effluent $\text{PO}_4\text{-P}$ concentration had increased from 0.2 to 27 mg l^{-1} over the 4-week period prior to sampling for the present study, and in Reactor 3, EBPR had gradually deteriorated over a 2-month period, leading to no net P removal and an effluent $\text{PO}_4\text{-P}$ concentration of 17.5 mg l^{-1} .

The biomass in all three reactors displayed anaerobic propionate uptake, PHA production and glycogen consumption followed by aerobic PHA degradation and glycogen production, as per the GAO phenotype. However, the cells were not targeted by GAOMIX or SBR9-1a probes. Some DF1MIX-hybridizing cells were present in Reactor 1 (see below), but most of the TFOs in the reactors were not targeted by these probes. Two rRNA gene clone libraries previously constructed from Reactor 3 biomass failed to identify the TFOs even though they comprised >50 % of all *Bacteria* (C. Yeates, personal communication). The first clone library used *Bacteria*-specific primers (27f and LS1608r; Lane, 1992) to amplify the 16S rRNA gene, intergenic spacer region and part of the 23S rRNA gene. The second library used 27f (Lane, 1992) and the *Alphaproteobacteria*-specific primer ALF969r, a modification of ALF968 (Neef *et al.*, 1999). ALF969 bound the abundant TFOs when used in FISH. Although 51 clones from 19 different operational taxonomic units (OTUs) were sequenced and analysed from these libraries, not one sequence was closely related to *Deftuvicoccus*, and none of the 16 *Alphaproteobacteria*-targeting FISH probes designed from the sequences bound abundant populations in the sludge (C. Yeates, personal communication). Therefore, SIP was used to specifically target the bacteria in the reactor capable of anaerobic propionate uptake to make the cloning more directed.

Characteristics of biomass used for SIP experiment

Fig. 1 shows the analysis from a cycle study carried out on Reactor 1 prior to sampling biomass for the SIP experiment. It demonstrates the anaerobic uptake of propionate and the cycling of intracellular PHA and glycogen during the anaerobic and aerobic periods, which are characteristics of the GAO phenotype.

Neither '*Candidatus Competibacter phosphatis*' nor the *Sphingomonas*-related putative GAOs (according to probe SBR9-1a) were detected in the biomass, which was dominated by *Alphaproteobacteria* ($61 \pm 14\%$ of all *Bacteria* by FISH quantification). Some of the *Alphaproteobacteria* bound the DF1MIX probes targeting a group of putative GAOs related to *D. vanus* (see below). However, many of the *Alphaproteobacteria* in the reactor did not bind these probes, suggesting that other putative GAOs belonging to the *Alphaproteobacteria* were present.

Fig. 1. A cycle study of Reactor 1 demonstrating the GAO phenotype of the sludge. The vertical line indicates the transition from the anaerobic to the aerobic period. ○, PHA; ●, glycogen; ▲, propionate.

Assessment of ^{13}C -labelling of nucleic acids and separation of labelled RNA from other nucleic acids

^{13}C -labelled propionate was supplied to the biomass over eight reactor cycles, but was only available under anaerobic conditions as no propionate was detected in any of the samples taken at the end of the anaerobic period. After the ^{13}C incubation, the $\delta^{13}\text{C}$ value of both DNA and RNA extracted from the biomass had increased (Table 1), indicating that bacteria capable of anaerobic propionate consumption had incorporated ^{13}C -labelled propionate. However, the $\delta^{13}\text{C}$ value in the RNA extracts was considerably higher than the $\delta^{13}\text{C}$ value in the DNA and the RNA was selected as the preferred nucleic acid for further analysis. This result demonstrates the advantage of using RNA-SIP compared to DNA-SIP, when the target organism is slow-growing but has a high metabolic activity.

Density-gradient centrifugation of RNA and subsequent fractionation of the gradient yielded 10 fractions, the density of which decreased linearly ($r^2 = 0.9911$) between 1.979 g ml^{-1} (fraction 1) and 1.872 g ml^{-1} (fraction 10).

Table 1. $\delta^{13}\text{C}$ values determined by isotope ratio mass spectrometry

Sample	$\delta^{13}\text{C}$ value (‰)
Glucose control	-26
Glucose + DNA before ^{13}C labelling	-29
Glucose + DNA after ^{13}C labelling	-1
Glucose + RNA before ^{13}C labelling	-28
Glucose + RNA after ^{13}C labelling	+643

An increase in the $\delta^{13}\text{C}$ value (more positive or less negative) indicates enrichment with ^{13}C in the nucleic acid.

RNA purified from each fraction was used to synthesize cDNA, which was then used as template in PCR amplification of near full-length 16S rRNA genes. PCR products were obtained from fractions 5, 6 and 8, but not from fraction 7, indicating that the separation of the ^{13}C -enriched RNA from the unlabelled RNA had been successful. As fraction 5 had the highest density, this fraction was the most ^{13}C -enriched and the PCR product from this fraction was used to construct a clone library.

Analysis of 16S rRNA sequences obtained from ^{13}C -labelled RNA

Eleven of 13 sequences (from the four most abundant OTUs by RFLP) were related to *D. vanus* (Maszenan *et al.*, 2005), within the *Alphaproteobacteria*. The remaining two sequences were related to *Gammaproteobacteria* and

Betaproteobacteria, respectively. Phylogenetic analysis of these sequences and published sequences showed that the sequences related to *D. vanus* formed a monophyletic group with two distinct clusters (Fig. 2). Cluster 1 comprised one sequence from the present study (Meyer Clone 17), *D. vanus*, four sequences from Wong *et al.* (2004) and four partial 16S rRNA gene sequences obtained from an EBPR system (T. Zhang *et al.*, unpublished; direct submission to GenBank). Cluster 2 was monophyletic and contained 10 of the 11 *Alphaproteobacteria* sequences obtained in this study, one sequence from a lab-scale anaerobic-aerobic operated bioreactor (Wong *et al.*, 2004) and three sequences obtained from a full-scale EBPR plant (K.D. McMahon *et al.*, unpublished; direct submission to GenBank).

The phylogenetic tree (Fig. 2) contains representatives from each of the orders within the *Alphaproteobacteria*.

Fig. 2. Phylogenetic tree (maximum-likelihood) of the sequences obtained and highly identical sequences obtained from GenBank. Clusters 1 and 2 indicate the two distinct phylogenetic *D. vanus* groups identified. Bootstrap values are indicated as a percentage of 1000 analyses. Clone sequences in Clusters 1 and 2 are presented as author and clone name followed by accession number. Sequences on dashed lines were added after phylogenetic analysis by the parsimony insertion tool of ARB and were between 311 and 962 nt in length. All other sequences were at least 1257 nt in length.

Table 2. Oligonucleotide probes designed for FISH. Helper probes are designated H

Probe	Probe sequence (5'–3')	Target site*	Formamide (%)†	Helper probe
DF988	GATACGACGCCCATGTCAAGGG	988–1009	35	H966 + H1038
DF1020	CCGGCCGAACCGACTCCC	1020–1037	35	H1038
H966	CTGGTAAGGTTCTGCGCGTTGC	966–987	–	–
H1038	AGCAGCCATGCAGCACCTGTGTGGCGT	1038–1064	–	–
H1205	AGCCAGCCCGTAAGGGCC	1205–1223	–	–

**E. coli* numbering of 16S rRNA gene.

†Used in FISH hybridization buffer.

The position of *Deffluvicoccus* within the *Acetobacteraceae* family is shown along with four other genera: *Acetobacter*, *Acidiphilum*, *Rubritepida* and *Roseococcus* (each represented by their type species). The last two genera (*Rubritepida* and *Roseococcus*) are the closest relatives of *D. vanus*.

Bootstrap values of 98 and 68 % support the separation of Clusters 1 and 2, and pair-wise comparison demonstrated the sequence identities were 93 % for Cluster 1 and 97 % for Cluster 2. The sequence identity between the two clusters was only 90 %, suggesting that each of the clusters may indeed comprise a separate species. It can be noted that clone AY351635 (Wong *et al.*, 2004) has a relatively long branch length. The identity of this sequence to the rest of the Cluster 1 sequences is only 93–95 %, whereas all other sequences within this cluster are at least 97 % identical.

Wong *et al.* (2004) designed two probes (TFO_DF218 and TFO_DF618, referred to as DF1MIX in this paper) that target sequences within Cluster 1 of the *D. vanus*-related organisms and showed these organisms to have some aspects of the GAO phenotype, but the research did not address the sequences within Cluster 2.

Optimization of FISH probes targeting Cluster 2

Two FISH probes were designed to target all the sequences in Cluster 2 of the *D. vanus*-related organisms: DF988 and DF1020 (Table 2). When used together, these probes are referred to as DF2MIX. Initial testing of the probes yielded low or no fluorescent signals even at low formamide concentrations (20 %). Weak binding of FISH probes can be caused by poor probe accessibility to the target site in the ribosome (Behrens *et al.*, 2003), but probe accessibility may be improved by unlabelled oligonucleotides or 'helper probes' which bind to the ribosome adjacent to or near the FISH probe target site (Fuchs *et al.*, 2000).

Three such helper probes were designed (Table 2) to target the 16S rRNA directly adjacent to the two FISH probes in loops 34 and 38 of the ribosome, as well as loop 35 positioned across from the DF988 probe site. Fig. 3 demonstrates the effect of the helper probes on the signal intensity of DF988 and DF1020. The addition of helper probe H1205

to the FISH had little effect on the signal intensity of either DF988 or DF1020, but helper probe H1038 led to a 2.8-fold increase in the intensity of DF988 and a 6.6-fold increase in the signal intensity of DF1020 (Fig. 3). Helper probe H966 led to a 3.4-fold increase in the signal intensity of DF988, but had little effect on the binding of DF1020. The combined use of helper probes H966 and H1038 with DF988 caused a 3.6-fold increase in signal intensity. In conclusion, we recommend that DF988 be used in conjunction with helper probes H966 and H1038, and that DF1020 be used in conjunction with helper probe H1038. Since the binding site of H966 overlaps with the target position of probe ALF968 (Neef *et al.*, 1999), when using DF988 (which requires H966), we suggest the use of ALF1b (Manz *et al.*, 1992) if simultaneous visualization of *Alphaproteobacteria* and DF988 organisms is required.

The dramatic improvement in the signal intensity through the use of helper probes stresses the importance of testing the effect of helper probes when new FISH probes are being optimized. In the present case, both probes would have been abandoned had helper probes not been designed.

Fig. 3. Pixel intensity (0–255) for probes DF988 (black bars) and DF1020 (grey bars) used alone or in combination with different unlabelled helper probes (H966, H1038 and H1205).

According to a BLAST search, no pure culture available in culture collections existed with fewer than seven mismatches to DF988 (*Craurococcus roseus*, in the *Alphaproteobacteria*) or 4 mismatches to DF1020 (*D. vanus*). Therefore, no pure culture positive or negative controls were available to evaluate the probe stringencies. Consequently, enriched biomass from Reactor 1 (from which the sequences had been obtained) was used as the positive control in optimization of the formamide concentration used in FISH. It should be noted, however, that there was no overlap in the binding of DF1MIX (which targets *D. vanus*) and DF2MIX in the samples where both groups of organisms were present (see below).

Increasing the formamide concentration from 35 to 40 % caused a 62 and 23 % decrease in signal intensity of the

DF988 and DF1020 probes, respectively. Thus, 35 % formamide was the highest formamide concentration that allowed binding of the probes to cells within the sample and we recommend that FISH with DF988 and DF1020 be carried out at this hybridization stringency.

Although all Cluster 2 sequences currently available contain both the DF988 and DF1020 target sequences, the probes appeared to have a slightly different specificity when tested. DF988 appeared to have a broader specificity than DF1020, as not all DF988-hybridizing cells were targeted by DF1020 (Fig. 4). Morphological diversity was also observed in the cells binding the probes. All cells were TFOs, but some formed very dense clusters of few cells and others formed clusters of more cells with a spatial arrangement that

Fig. 4. FISH images of the biomass from Reactor 1 (used in the SIP experiment). The image demonstrates the difference in specificity of probes DF988 and DF1020. Each channel is shown separately: (a) blue, all *Bacteria* (EUBMIX); (b) red, DF988, (c) green, DF1020. (d) An overlay of all three probes where organisms targeted by all probes are white, organisms targeted only by DF988 are magenta and other bacteria are blue. In this image, there are no cyan-coloured cells which would have been targeted only by DF1020. Scale bar, 10 μ m.

appeared to leave space between individual cells when observed with FISH (Fig. 4). These results indicate that the phylogenetic diversity of Cluster 2 is yet to be fully described as more sequence information becomes available.

Phenotype and abundance of Cluster 2

The ability of Cluster 2 *D. vanus*-related organisms (DF2MIX-hybridizing cells) to demonstrate the GAO phenotype was partially confirmed by their anaerobic production of PHA and aerobic degradation of PHA as shown in Fig. 5. The images show that Cluster 2 organisms contained PHA inclusions at the end of the anaerobic period, but lacked intracellular PHA at the end of the aerobic period. The cells had a tetrad morphology, which was not always apparent from the FISH images, but very clear when observing the cells with transmitted light microscopy (Fig. 5).

Cluster 2 *D. vanus*-related organisms accounted for $33 \pm 9.2\%$ of all *Bacteria* in biomass from Reactor 1 (Fig. 6a) and were even more abundant in two other lab-scale SBRs configured for EBPR, although P removal had deteriorated. Cluster 2 organisms accounted for 51 ± 16.4 and $55 \pm 13.2\%$ of all *Bacteria* in Reactors 2 and 3, respectively (Fig. 6b and c). In both Reactors 2 and 3, the high abundance of Cluster 2 *D. vanus*-related organisms may have contributed to the deterioration of P removal performance. The lab-scale reactors found to be highly enriched in Cluster 2 organisms were operated with propionate as the sole carbon source. The use of propionate has recently been suggested as a method of favouring the growth of PAOs in lab-scale reactors (Chen *et al.*, 2005; Pijuan *et al.*, 2004); however, the abundance of the Cluster 2 organisms in several propionate-fed reactors challenges this hypothesis and stresses the need to further investigate the diversity of GAOs.

The Cluster 2 *D. vanus*-related organisms were also found in biomass from full-scale plants A and B (Fig. 6d–f) sampled in January 2005, although their abundance was low (estimated $<5\%$ of biomass). Five additional Plant A samples obtained during an earlier 10 month study were also screened for Cluster 2 *D. vanus*-related organisms, but they were only detected in one sample. The significance of Cluster 2 *D. vanus*-related organisms in full-scale EBPR is thus not clear, but their presence indicates that they may play a role in wastewater treatment. Future studies comparing the abundance of Cluster 1 and Cluster 2 *D. vanus*-related organisms, '*Candidatus Competibacter phosphatis*' and '*Candidatus Accumulibacter phosphatis*' with the P removal performance evaluated by anaerobic VFA uptake rate to P release rate are needed to explore more fully the roles of these organisms in EBPR and its deterioration.

The identification of putative GAOs in the two *D. vanus* clusters poses the question as to whether *D. vanus* is the first isolate of a GAO. *D. vanus* has not been tested in pure culture under anaerobic/aerobic cycling to determine if it has the GAO phenotype (Maszenan *et al.*, 2005). The answer

Fig. 5. FISH images of the biomass from Reactor 1 (a–d) and Reactor 2 (e–h). In (a), (c), (e) and (g), Cluster 2 *D. vanus* cells are magenta (overlay of red DF2MIX and blue EUBMIX) and other *Bacteria* are blue (blue EUBMIX). In (b), (d), (f) and (h), overlays were of phase-contrast (black and white) images and Nile-Blue-stained PHAs in red. The same fields are presented in the left and right panels. The samples were taken at the end of the anaerobic (a and b; e and f) and at the end of the aerobic (c and d; g and h) periods. Scale bar, 10 μ m.

to this question would be of interest to the exploration of the metabolic characteristics of the *Defluvicoccus* group.

Conclusion

This study found that Cluster 2 *D. vanus*-related organisms are putative GAOs because they produce PHA anaerobically

Fig. 6. FISH images of the biomass from Reactor 1 used in the SIP experiment (a), Reactor 2 (b), Reactor 3 (c) (the latter two were both lab-scale anaerobic–aerobic SBRs in which P removal had deteriorated), Plant A (d and e) and Plant B (f) (the latter two were both full-scale wastewater treatment plants from Southeast Queensland, Australia). In all images, magenta-coloured cells are Cluster 2 *D. vanus* (overlay of red DF2MIX and blue EUBMIX) and blue-coloured cells are other *Bacteria* (blue EUBMIX). In (a) cyan-coloured cells are Cluster 1 *D. vanus* (overlay of green DF1MIX and blue EUBMIX) and in (b) cyan-coloured cells are '*Candidatus Accumulibacter phosphatis*' (overlay of green PAOMIX and blue EUBMIX). Scale bar, 10 μ m.

and they utilize it aerobically in complex microbial communities where EBPR is not occurring. Their presence was detected in both lab-scale and full-scale wastewater treatment processes being operated for EBPR.

Our data suggest that the Cluster 2 *D. vanus*-related organisms may be of particular significance to deterioration of EBPR when propionate is a carbon source. We showed that RNA-SIP could be used to specifically target these organisms, although conventional cloning procedures had failed to do so even in a highly enriched biomass. This method thus holds the potential to capture the diversity of putative PAOs and GAOs in more complex microbial environments,

e.g. full-scale wastewater treatment plants. Identification of putative GAOs and the development of monitoring tools is a prerequisite for understanding the disturbance they cause to EBPR. The FISH probes developed in the present study provide a tool to identify and quantify a group of putative GAOs not previously identified and constitute a step in the way of knowing and studying more organisms that positively or negatively impact on EBPR.

ACKNOWLEDGEMENTS

We thank the Environmental Biotechnology Cooperative Research Centre, Australia, and the Danish Natural Science Research Council for

funding this work. Adrian Oehmen operated Reactor 1, Hua Bing Lui operated Reactor 2 and Romain Lemaire operated Reactor 3. Thanks to Christine Yeates for assisting in clone library construction and analysis and Mike Manefield at University of New South Wales, Australia, for helpful advice in adapting the RNA-SIP method.

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