Anaerobic and aerobic metabolism of glycogen-accumulating organisms selected with propionate as the sole carbon source

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In the microbial competition observed in enhanced biological phosphorus removal (EBPR) systems, an undesirable group of micro-organisms known as glycogen-accumulating organisms (GAOs) compete for carbon in the anaerobic period with the desired polyphosphate-accumulating organisms (PAOs). Some studies have suggested that a propionate carbon source provides PAOs with a competitive advantage over GAOs in EBPR systems; however, the metabolism of GAOs with this carbon source has not been previously investigated. In this study, GAOs were enriched in a laboratory-scale bioreactor with propionate as the sole carbon source, in an effort to better understand their biochemical processes. Based on comprehensive solid-, liquid- and gas-phase chemical analytical data from the bioreactor, a metabolic model was proposed for the metabolism of propionate by GAOs. The model adequately described the anaerobic stoichiometry observed through chemical analysis, and can be a valuable tool for further investigation of the competition between PAOs and GAOs, and for the optimization of the EBPR process. A group of Alphaproteobacteria dominated the biomass (96 % of Bacteria) from this bioreactor, while post-fluorescence *in situ* hybridization (FISH) chemical staining confirmed that these Alphaproteobacteria produced poly- β -hydroxyalkanoates (PHAs) anaerobically and utilized them aerobically, demonstrating that they were putative GAOs. Some of the Alphaproteobacteria were related to *Deffluvicoccus vanus* (16 % of Bacteria), but the specific identity of many could not be determined by FISH. Further investigation into the identity of other GAOs is necessary.

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INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is a widespread activated sludge process for the removal of phosphorus from wastewater. Sequential anaerobic and aerobic phases are employed for the enrichment of a group

of bacteria known as polyphosphate-accumulating organisms (PAOs), which are primarily responsible for EBPR. Another group of bacteria, glycogen-accumulating organisms (GAOs), also proliferate in systems with sequential anaerobic and aerobic phases, competing with PAOs for the often limiting carbon substrates (Bond *et al.*, 1998; Satoh *et al.*, 1994; Thomas *et al.*, 2003; Whang & Park, 2002). Currently, there are no pure cultures of PAOs or GAOs (Seviour *et al.*, 2003), so their biochemical transformations have been determined through chemical analyses of enriched laboratory-scale mixed EBPR microbial communities. Metabolic models have been proposed to describe the biochemical transformations occurring in PAOs consuming acetate (Smolders *et al.*, 1994a, b, 1995) and propionate (Oehmen *et al.*, 2005b) as the sole carbon source. Similarly, GAO enrichments consuming acetate have also been modelled (Filipe *et al.*, 2001a; Zeng *et al.*, 2002, 2003b). These metabolic models provide useful tools for optimization of EBPR and for research into the competition between PAOs and GAOs.

Under anaerobic conditions, both PAOs and GAOs can take up volatile fatty acids (VFAs) and convert them into

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Abbreviations: acetyl-CoA*, activated acetyl-CoA; EBPR, enhanced biological phosphorus removal; FISH, fluorescence *in situ* hybridization; GAO, glycogen-accumulating organism; PAO, polyphosphate-accumulating organism; PHA, poly- β -hydroxyalkanoate; PHB, poly- β -hydroxybutyrate; PH2MV, poly- β -hydroxy-2-methylvalerate; PHV, poly- β -hydroxyvalerate; propionyl-CoA*, activated propionyl-CoA; SBR, sequencing batch reactor; TFO, tetrad-forming organism; TOGA, titration and off-gas analysis; VFA, volatile fatty acid.

intracellular poly- β -hydroxyalkanoates (PHAs). To obtain the energy for anaerobic VFA uptake, the metabolic models propose that PAOs hydrolyse intracellularly stored polyphosphate and glycogen, while GAOs hydrolyse only glycogen for this purpose, as they have no stored polyphosphate. Under aerobic conditions, PAOs and GAOs oxidize the intracellular PHA to grow and to replenish their stored glycogen. PAOs also use a portion of the energy generated from PHA oxidation for orthophosphate uptake and synthesis of intracellular polyphosphate. Since GAOs consume the limited VFAs in the anaerobic period without contributing to phosphorus removal, they are highly undesirable microorganisms in EBPR systems.

Culture-independent methods have been used to identify a PAO, named '*Candidatus Accumulibacter phosphatis*' (*Accumulibacter*), which is a member of the family *Rhodocyclaceae* within the Betaproteobacteria (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999). Fluorescence *in situ* hybridization (FISH) has shown *Accumulibacter* to be an abundant organism in laboratory-scale EBPR cultures with various carbon sources (Levantesi *et al.*, 2002; Liu *et al.*, 2001; Oehmen *et al.*, 2004, 2005b; Onda *et al.*, 2002; Pijuan *et al.*, 2004; Zeng *et al.*, 2003a). *Accumulibacter* has also been reported in full-scale EBPR systems (Saunders *et al.*, 2003; Zilles *et al.*, 2002). A similar approach has identified deeply branching members of the Gammaproteobacteria (Nielsen *et al.*, 1999) as putative GAOs. These organisms have been called either '*Candidatus Competibacter phosphatis*' (henceforth called *Competibacter*; Crocetti *et al.*, 2002) or the GB lineage (Kong *et al.*, 2002). *Competibacter* can consume acetate as the sole carbon source, but a recent study has shown that they take up propionate very slowly (Oehmen *et al.*, 2005a), and thus tend to be out-competed by *Accumulibacter* (Oehmen *et al.*, 2005a, 2006; Pijuan *et al.*, 2004).

Tetrad-forming organisms (TFOs; also called 'G-bacteria') have been linked to the deterioration of EBPR in laboratory-scale systems. Using culture-independent methods, two distinct groups of TFOs have recently been identified as members of the Alphaproteobacteria and shown to be putative GAOs. One group are members of the order Sphingomonadales, and the other is related to the isolate *Deffluvicoccus vanus* within the order Rhodospirillales. Both of these organisms could consume acetate as the sole carbon source (Beer *et al.*, 2004; Wong *et al.*, 2004), but the *D. vanus*-related organism has also been recently demonstrated to consume propionate (Meyer *et al.*, 2006), unlike *Competibacter*.

This study investigated the metabolism of an enriched culture of Alphaproteobacteria TFOs that were fed with propionate as the sole carbon source and demonstrated the GAO phenotype. Anaerobic and aerobic biochemical transformations as well as maintenance processes are described in detail from experimental data, and a metabolic model describing propionate uptake by GAOs is proposed.

METHODS

Reactor setup and operation. An enrichment of GAOs was obtained through operation of a sequencing batch reactor (SBR) for a period of 5 months. The SBR had a working volume of 8 l and was operated with 6 h cycles, beginning with a 130 min anaerobic period, followed by a 160 min aerobic period and a 70 min settle and decant period. Two litres of synthetic wastewater (Smolders *et al.*, 1994b; Zeng *et al.*, 2003b) with propionate as the sole carbon source were fed during the first 6 min of the anaerobic period in each cycle, resulting in a hydraulic retention time (HRT) of 24 h. The sludge retention time (SRT) was approximately 8 days, with 250 ml mixed liquor being wasted at the end of each aerobic period. The pH was maintained at 7.0 ± 0.1 during the anaerobic and aerobic phases using a one-way controller that dosed 0.5 M HCl when the pH was above the setpoint. The reactor was seeded with sludge from an SBR enriched for PAOs, as reported in Oehmen *et al.* (2005b), and maintained under identical operational conditions apart from a considerably reduced level of phosphate in the feed (2 mg $\text{PO}_4\text{-P l}^{-1}$ instead of 53 mg $\text{PO}_4\text{-P l}^{-1}$) to select for GAOs, where $\text{PO}_4\text{-P}$ is phosphate.

Cycle studies and batch experiments. The performance of the SBR was monitored through cycle studies in which samples were collected at various points throughout a cycle and chemically analysed. Propionate, PHA, glycogen, orthophosphate and ammonia were analysed (Oehmen *et al.*, 2005b) every 10 min for the first 40 min of the anaerobic period, followed by 20 min intervals thereafter. In the subsequent aerobic phases, PHA, glycogen, orthophosphate and ammonia analyses were carried out every 20–30 min. Total suspended solids (TSS) and volatile suspended solids (VSS) samples were determined at the end of each aerobic period (APHA, AWWA & WPCF, 1995).

Oxygen consumption and CO_2 production during a cycle were determined via the titration and off-gas analysis (TOGA) sensor with 2 h anaerobic and 3 h aerobic phases (Pratt *et al.*, 2003).

The anaerobic hydrogen ion production (or consumption) found in each test with the TOGA sensor is caused by two main factors. Propionic acid uptake by GAOs leads to the consumption of H^+ , and CO_2 production has a pH effect through dissolving in the liquid and influencing the bicarbonate acid-base system. The total amount of hydrogen ions produced (HP_{total}) can be determined as:

$$\text{HP}_{\text{total}} = \frac{\text{propionate}_{\text{uptake}}}{3} + \frac{(\text{CO}_{2\text{produced}} - \text{CO}_{2\text{transfer}})}{1 + 10^{\text{pK}_a - \text{pH}_{\text{op}}}} \quad (1)$$

where the CO_2 produced minus the CO_2 transferred to the gas phase is equal to the CO_2 dissolved in the liquid. The operational pH (pH_{op}) is maintained at 7.0 in these tests and the pK_a is 6.35 for the bicarbonate acid-base buffering system. The anaerobic CO_2 production can therefore be calculated as:

$$\text{CO}_{2\text{produced}} = (1 + 10^{\text{pK}_a - \text{pH}_{\text{op}}}) \left(\text{HP}_{\text{total}} - \frac{\text{propionate}_{\text{uptake}}}{3} + \frac{\text{CO}_{2\text{transfer}}}{1 + 10^{\text{pK}_a - \text{pH}_{\text{op}}}} \right) \quad (2)$$

where the CO_2 transfer and hydrogen ion production are measured through the TOGA sensor, and the propionate uptake is measured through off-line samples. The aerobic CO_2 production was determined in a similar manner to anaerobic CO_2 production, where the HP_{total} is influenced by ammonium uptake for biomass growth instead of

propionate uptake:

$$\text{CO}_{2\text{produced}} = \left(1 + 10^{\text{pK}_a - \text{pH}_{\text{op}}}\right) \times \left(\text{HP}_{\text{total}} - \text{ammonium}_{\text{uptake}} + \frac{\text{CO}_{2\text{transfer}}}{1 + 10^{\text{pK}_a - \text{pH}_{\text{op}}}}\right) \quad (3)$$

A more detailed description of the TOGA and the determination of CO₂ production has been reported in the literature (Oehmen *et al.*, 2005b; Pratt *et al.*, 2003; Zeng *et al.*, 2003b).

Determination of the anaerobic and aerobic maintenance coefficients. The rate of glycogen hydrolysis, PHA accumulation and CO₂ production incurred by anaerobic maintenance was determined through an 8 h anaerobic batch test without propionate addition. The aerobic maintenance was found through extending the aerobic period to 9 h in one of the TOGA tests described above for the determination of oxygen uptake and CO₂ production. All other operational procedures in the anaerobic and aerobic maintenance batch tests were identical to those described above.

Analytical procedures. Orthophosphate, ammonia, nitrate and nitrite were analysed using a QuikChem8000 (Lachat, WI) flow injection analyser (FIA). Propionic acid was measured by HPLC with an HPX-87H 300 × 7.8 mm Bio-Rad Aminex ion exclusion HPLC column operated at 65 °C. FIA and propionic acid samples were obtained through filtering mixed liquor from the SBR using 0.22 µm Millex GP syringe-driven filters. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined in accordance with standard methods (APHA, AWWA & WPCF *et al.*, 1995). Glycogen and PHA analyses were performed as reported in Oehmen *et al.* (2005b). Elemental analysis of the enriched biomass was performed using 0.5 mg lyophilized sludge in a Perkin-Elmer 240 Elemental Analyser. The carbon, hydrogen, oxygen and nitrogen measurements were used to determine the active biomass composition, after subtracting the PHA and glycogen components stored in the sludge.

FISH was carried out as detailed in Amann (1995), and a list of all oligonucleotide probes used during this study is shown in Table 1.

Table 1. Oligonucleotide probes used during this study

Helper probes are designated with an H; competitor probes are designated with a c.

Probe	Probe sequence (5'–3')	FA* concn (%)	Reference
EUBMIX for all Bacteria (in equal amounts)			
EUB I	GCT GCC TCC CGT AGG AGT	35	Amann <i>et al.</i> (1990)
EUB II	GCA GCC ACC CGT AGG TGT	35	Daims <i>et al.</i> (1999)
EUB III	GCT GCC ACC CGT AGG AGT	35	Daims <i>et al.</i> (1999)
ALF1b for some Alphaproteobacteria			
	CGT TCG (C/T) TC TGA GCC AG	20	Manz <i>et al.</i> (1992)
ALF969 for some Alphaproteobacteria and to be used with c969a and c969b			
	TGG TAA GGT TCT GCG CGT	35	R. Lemaire and others, unpublished results
SBR9-1a for <i>Sphingomonas</i>-related organisms			
	AAG CGC AAG TTC CCA GGT TG	30	Beer <i>et al.</i> (2004)
DF1MIX for cluster 1 <i>D. vanus</i> (in equal amounts)			
TFO_DF218	GAA GCC TTT GCC CCT CAG	35	Wong <i>et al.</i> (2004)
TFO_DF618	GCC TCA CTT GTC TAA CCG	35	Wong <i>et al.</i> (2004)
DF2MIX for cluster 2 <i>D. vanus</i> (to be used with H966 and H1038 and in equal amounts)			
DF988	GAT ACG ACG CCC ATG TCA AGG G	35	Meyer <i>et al.</i> (2006)
DF1020	CCG GCC GAA CCG ACT CCC	35	Meyer <i>et al.</i> (2006)
GAOMIX for <i>Competibacter phosphatis</i> (to be used with cGB_G2 and in equal amounts)			
GAOQ989	TTC CCC GGA TGT CAA GGC	35	Crocetti <i>et al.</i> (2002)
GB_G2	TTC CCC AGA TGT CAA GGC	35	Kong <i>et al.</i> (2002)
Other			
c969a	AGG TAA GGT TCT GCG CGT		R. Lemaire and others, unpublished results
c969b	GGG TAA GGT TCT GCG CGT		R. Lemaire and others, unpublished results
cGB_G2	TTC CCC GGA TGT CAA GGC		Kong <i>et al.</i> (2002)
H966	CTG GTA AGG TTC TGC GCG TTG C		Meyer <i>et al.</i> (2006)
H1038	AGC AGC CAT GCA GCA CCT GTG TGG CGT		Meyer <i>et al.</i> (2006)

*FA, formamide (used in FISH buffer).

ALF1b (Manz *et al.*, 1992) and ALF969 (R. Lemaire and others, unpublished results) probes were used for Alphaproteobacteria. ALF969 was modified from ALF968 (Neef, 1997) because *Competibacter* and *Kouleothrix* (a filamentous organism commonly found in activated sludge systems (Beer *et al.*, 2002) are perfectly targeted by ALF968. Competitor probes targeting *Competibacter* and *Kouleothrix* were added without a fluorescent label to ensure the differentiation of these organisms from Alphaproteobacteria.

The probed biomass was examined using a Zeiss LSM510 Meta confocal laser scanning microscope. Quantification was done using previously reported image analysis techniques (Bouchez *et al.*, 2000; Crocetti *et al.*, 2002), in which the area of cells binding the specific probe is expressed as the mean percentage of the area of the entire bacterial community (EUBMIX, Table 1). The standard error of the mean (SEM) was calculated as the standard deviation of the area percentage divided by the square root of the number of images ($n=51$). Post-FISH chemical staining was carried out as detailed by Crocetti *et al.* (2002) using Nile Blue to stain for PHA (Ostle & Holt, 1982). Samples from the end of both the anaerobic and aerobic periods were stained to confirm intracellular PHA cycling.

RESULTS AND DISCUSSION

SBR operation

A cycle study of the SBR that illustrates the typical steady-state operation is shown in Fig. 1. The GAO phenotype is clearly displayed: VFA uptake, PHA accumulation and glycogen degradation under anaerobic conditions; and PHA oxidation and glycogen replenishment under aerobic conditions. There was no phosphorus release observed in the SBR after the first month of operation. Nitrification inhibition was achieved through addition of allyl-*N*-thiourea, and confirmed by the absence of NO_x (below the detection limit) throughout the reactor operation. The primary fractions of PHA formed through propionate uptake were poly- β -hydroxyvalerate (PHV) and poly- β -hydroxy-2-methylvalerate (PH2MV). This differs from GAOs enriched with acetate, which produce primarily

poly- β -hydroxybutyrate (PHB) and PHV (Filipe *et al.*, 2001a; Zeng *et al.*, 2003b).

FISH and post-FISH chemical staining

FISH quantification showed that 96 % of all bacteria in the SBR (SEM = 1 %) were Alphaproteobacteria (ALF969). The cells hybridizing the ALF969 probe also hybridized the ALF1b probe, confirming the specificity of the ALF969 probe. Morphologically, almost all of the Alphaproteobacteria were TFOs; other morphologies were only observed in very low abundance. Most of the Alphaproteobacteria observed using FISH (Fig. 2A) contained PHA at the end of the anaerobic period (Fig. 2B), and the PHA was mostly absent from the cells at the end of the aerobic phase (data not shown). The anaerobic-aerobic cycling of PHA in the Alphaproteobacteria cells combined with their abundance in the biomass strongly supports the hypothesis that they were putative GAOs.

Competibacter (GAOMIX, Table 1) and *Sphingomonas*-related organisms (SBR9-1a) were not detected in the biomass. DF1MIX (Table 1) *Deffluvicoccus*-related organisms accounted for 8 % (SEM = 1 %) of the Bacteria. DF2MIX (Table 1) *Deffluvicoccus*-related organisms accounted for an additional 8 % (SEM = 1 %) of the Bacteria, and the cells that bound these probes were TFOs. The fact that the majority of Alphaproteobacteria in the biomass that cycled PHA did not bind the current probes for GAOs strongly suggests the presence of other GAOs, despite the similar morphology of these organisms to those described in the literature (Beer *et al.*, 2004; Meyer *et al.*, 2006; Wong *et al.*, 2004). It may be that the probes from these publications do not target the diversity of the organisms within these already identified groups so that additional sequences are required in order to broaden the probe specificities; alternatively, another group of GAOs unrelated to *Sphingomonas* or *D. vanus* may exist.

Fig. 1. Cycle study showing VFA, PHA, glycogen and ammonia transformations from a typical cycle in the SBR. The GAO phenotype of anaerobic VFA uptake, and anaerobic-aerobic cycling of PHA and glycogen is clearly displayed.

Fig. 2. Images of the same microscopic field of biomass taken from the end of the anaerobic period. (A) FISH showing Alphaproteobacteria (ALF969; magenta) and all other Bacteria (EUBMIX; blue), and (B) post-FISH staining with Nile Blue for PHA (red). PHA was not observed in biomass samples taken from the end of aerobic period (not shown). Thus, these Alphaproteobacteria cells are putative GAOs as anaerobic–aerobic cycling of PHA was observed. Bars, 10 μm .

A metabolic model for propionate uptake by GAOs

A metabolic model has been formulated as outlined below to describe the anaerobic metabolism of propionate by GAOs, and is presented schematically in Fig. 3. Propionate is taken up anaerobically by GAOs and converted to propionyl-CoA, using glycogen hydrolysis as the sole energy source. Glycogen glycolysis to pyruvate generates energy in the form of ATP, proceeding through what is hypothesized to be

the Embden–Meyerhof (EM) pathway (Filipe *et al.*, 2001a). A portion of the pyruvate is converted to acetyl-CoA and CO_2 , which produces reducing equivalents in the form of NADH_2 . The remainder of the pyruvate is converted to propionyl-CoA (consuming NADH_2 in the process) such that the reduction-oxidation (redox) balance is maintained within the cells. Acetyl-CoA and propionyl-CoA are then reduced to form activated acetyl-CoA (acetyl-CoA*) and activated propionyl-CoA (propionyl-CoA*), the precursors of PHA. Assuming acetyl-CoA and propionyl-CoA are

Fig. 3. Schematic of the proposed metabolism of propionate (PA) by GAOs, adapted from Zeng *et al.* (2003b), showing consumption and production of components. All parameters are presented on a C-mol basis.

randomly condensed, the PHA composition is calculated based on the probability of these molecules combining together in a large pool of acetyl-CoA and propionyl-CoA (Filipe *et al.*, 2001a). Selective condensation proposes that all of the acetyl-CoA will preferentially bind to propionyl-CoA to form PHV, while the remainder of the propionyl-CoA molecules are condensed to PH2MV, resulting in no PHB production (Oehmen *et al.*, 2005b). The experimental results are compared later to the model predictions for both random and selective condensation of acetyl-CoA and propionyl-CoA.

A model describing the aerobic processes of GAOs has been developed previously by Zeng *et al.* (2003b). This model may also be applied when propionate is the carbon source fed anaerobically. The change in carbon source only affects the fractions of acetyl-CoA and propionyl-CoA metabolized aerobically. These parameters may be obtained from the anaerobic model stoichiometry.

GAO metabolic model reactions

r_1 , propionate is taken up into the cell and converted to propionyl-CoA, where α_{GAO} is defined as the energy required for transport of one carbon-mole (C-mol) of VFA across the cell membrane, which has been shown to be pH dependent (Filipe *et al.*, 2001a; Smolders *et al.*, 1994b):

r_2 , glycogen is hydrolysed via the EM pathway (Gottschalk, 1986):

r_3 , acetyl-CoA production from pyruvate through oxidative decarboxylation (Voet & Voet, 1990):

r_4 , propionyl-CoA production from pyruvate through the propionate-succinate pathway (Gottschalk, 1986):

r_5 , activated acetyl-CoA (acetyl-CoA*) production (Filipe *et al.*, 2001a):

r_6 , activated propionyl-CoA (propionyl-CoA*) production (Filipe *et al.*, 2001a):

The aforementioned internal reactions may be expressed in terms of the measurable parameters as shown below:

$$r_{\text{propionate}} = -r_1$$

$$r_{\text{glycogen}} = -r_2$$

$$r_{\text{CO}_2} = r_3$$

$$r_{\text{pyruvate}} = 2r_2 - r_3 - r_4$$

$$r_{\text{acetyl-CoA}^*} = r_5$$

$$r_{\text{propionyl-CoA}^*} = r_6$$

$$r_{\text{NADH}_2} = 2r_2 + r_3 - 2r_4 - 0.5r_5 - 0.5r_6$$

$$r_{\text{ATP}} = -(1 + 3\alpha_{\text{GAO}})r_1 + 3r_2$$

$$r_{\text{acetyl-CoA}} = r_3 - r_5$$

$$r_{\text{propionyl-CoA}} = r_1 + r_4 - r_6$$

$$r_{\text{H}_2\text{O}} = r_1 - r_2 + 2r_4$$

Assuming that there is no accumulation of energy, reducing power, acetyl-CoA, propionyl-CoA and pyruvate then $r_{\text{NADH}_2} = 0$, $r_{\text{ATP}} = 0$, $r_{\text{acetyl-CoA}} = 0$, $r_{\text{propionyl-CoA}} = 0$ and $r_{\text{pyruvate}} = 0$. The internal and measurable reactions are then resolved with respect to propionate uptake, where the energy required for transport of 1 C-mol of propionate across the GAO cell membrane is defined as the parameter α_{GAO} :

$$r_2 = (1/3 + \alpha_{\text{GAO}})r_1$$

$$r_3 = (1/2 + \alpha_{\text{GAO}})r_1$$

$$r_4 = (1/6 + \alpha_{\text{GAO}})r_1$$

$$r_5 = (1/2 + \alpha_{\text{GAO}})r_1$$

$$r_6 = (7/6 + \alpha_{\text{GAO}})r_1$$

$$r_{\text{propionate}} = -r_1$$

$$r_{\text{glycogen}} = -(1/3 + \alpha_{\text{GAO}})r_1$$

$$r_{\text{CO}_2} = (1/2 + \alpha_{\text{GAO}})r_1$$

$$r_{\text{acetyl-CoA}^*} = (1/2 + \alpha_{\text{GAO}})r_1$$

$$r_{\text{propionyl-CoA}^*} = (7/6 + \alpha_{\text{GAO}})r_1$$

$$r_{\text{H}_2\text{O}} = (1 + \alpha_{\text{GAO}})r_1$$

Therefore, the following overall relationship can be obtained (mole basis):

$$\begin{aligned} & -\text{Propionate} - \left(\frac{1}{2} + \alpha_{\text{GAO}}\right)\text{glycogen} \\ & + (1 + \alpha_{\text{GAO}})\text{H}_2\text{O} + \left(\frac{1}{2} + \alpha_{\text{GAO}}\right)\text{CO}_2 \end{aligned} \quad (4)$$

$$\begin{aligned}
& + \left(\frac{1}{2} + \alpha_{\text{GAO}} \right) \text{Acetyl-CoA}^* \\
& + \left(\frac{7}{6} + \alpha_{\text{GAO}} \right) \text{Propionyl-CoA}^* = 0
\end{aligned} \quad (5)$$

Assuming that acetyl-CoA* and propionyl-CoA* are randomly condensed as PHB, PHV and PH2MV, as reported in Filipe *et al.*, then the overall equation is shown below (C-mol basis):

$$\begin{aligned}
& - \text{CH}_2\text{O}_{2/3} - (2/3 + 2\alpha_{\text{GAO}})\text{CH}_{10/6}\text{O}_{5/6} \\
& + (1/3 + \alpha_{\text{GAO}}/3)\text{H}_2\text{O}
\end{aligned} \quad (6)$$

$$\begin{aligned}
& + \frac{2(0.5 + \alpha_{\text{GAO}})^2}{3(5/3 + 2\alpha_{\text{GAO}})} \text{CH}_{1.5}\text{O}_{0.5} \\
& + \frac{5(0.5 + \alpha_{\text{GAO}})(7/6 + \alpha_{\text{GAO}})}{3(5/3 + 2\alpha_{\text{GAO}})} \text{CH}_{1.6}\text{O}_{0.4}
\end{aligned} \quad (7)$$

$$+ \frac{(7/6 + \alpha_{\text{GAO}})^2}{(5/3 + 2\alpha_{\text{GAO}})} \text{CH}_{5/3}\text{O}_{1/3} + (1/6 + \alpha_{\text{GAO}}/3)\text{CO}_2 = 0 \quad (8)$$

If selective condensation of acetyl-CoA* and propionyl-CoA* occurs as detailed in Oehmen *et al.* (2005b), then there is no PHB production, and the overall equation is adjusted to:

$$\begin{aligned}
& - \text{CH}_2\text{O}_{2/3} - (2/3 + 2\alpha_{\text{GAO}})\text{CH}_{10/6}\text{O}_{5/6} \\
& + (1/3 + \alpha_{\text{GAO}}/3)\text{H}_2\text{O}
\end{aligned} \quad (9)$$

$$\begin{aligned}
& + (5/6 + 5/3\alpha_{\text{GAO}})\text{CH}_{1.6}\text{O}_{0.4} + 2/3\text{CH}_{5/3}\text{O}_{1/3} \\
& + (1/6 + \alpha_{\text{GAO}}/3)\text{CO}_2 = 0
\end{aligned} \quad (10)$$

Characterizing the anaerobic stoichiometry of propionate-enriched GAOs

The anaerobic biochemical transformations found experimentally in this study are summarized in Table 2, and

compared to the model predictions. Glycogen, PHA and VFA were analysed through off-line samples as described above. The anaerobic CO₂ production was found through equation (2) using the CO₂ transfer and hydrogen ion production data obtained through the TOGA sensor (shown in Fig. 4), in conjunction with the propionate uptake. From the experimental results shown in Table 2, a carbon recovery of 97.8 % was achieved through a carbon balance, while a redox balance yielded 99.5 % recovery. Both results support the accuracy of the experimental data and strongly suggest that all relevant compounds were measured in the experiments.

In Table 2, a close fit can be observed between the experimental data and the metabolic model based on random condensation of acetyl-CoA and propionyl-CoA. This suggests that the proposed model adequately describes the stoichiometry associated with propionate uptake by GAOs. In contrast to the findings for GAOs, the stoichiometry of PAOs fed with propionate has been found to correlate closely with the model based on selective condensation of acetyl-CoA and propionyl-CoA (Oehmen *et al.*, 2005b). The reason for this observed difference in PHA formation between PAOs (selective) and GAOs (random) is unclear at present, but seems to indicate a slight, yet distinctive, difference between the metabolic pathways of these organisms.

The energy required for transport of 1 C-mol of propionate across the GAO cell membrane is defined as the parameter α_{GAO} , which is dependent on the ambient pH of the system (Filipe *et al.*, 2001a; Smolders *et al.*, 1994b). The model predictions in Table 2 represent the case when α_{GAO} equals zero. Filipe *et al.* (2001a) estimated an α_{GAO} of 0.06 at a pH of 7.0 for an acetate-enriched GAO culture. Further investigation into the estimation of the α_{GAO} parameter for GAOs would be beneficial in order to support the hypothesis that α_{GAO} does indeed equal zero at a pH of 7.0, as suggested by this study.

Aerobic stoichiometry

The aerobic stoichiometry observed in this study is summarized in Table 3. The carbon balance closes to

Table 2. Anaerobic stoichiometry associated with propionate uptake by GAOs: model predictions and experimental results, in comparison with PAO and GAO studies from the literature

All units are C-mol C-mol⁻¹. Experimental results shown are the mean obtained from five separate cycle studies, except for CO₂ production, which was obtained through averaging results from three TOGA tests. Results were calculated from the ratio of the carbon transformation rates during the period of propionate uptake.

Model or experiment (reference)	Gly/VFA	CO ₂ /VFA	PHB/VFA	PHV/VFA	PH2MV/VFA	PHA/VFA
Model, selective	0.67	0.17	0.00	0.83	0.67	1.50
Model, random	0.67	0.17	0.10	0.58	0.82	1.50
Experimental results	0.66	0.20	0.05	0.64	0.81	1.50
Experimental SD	0.03	0.03	0.01	0.03	0.03	0.05
Propionate PAO model (Oehmen <i>et al.</i> , 2005b)	0.33	0.11	0	0.56	0.67	1.22
Acetate GAO model (Zeng <i>et al.</i> , 2002)	1.12	0.27	1.36	0.46	0.04	1.86
Acetate PAO model (Smolders <i>et al.</i> , 1994b)	0.50	0.17	1.33	0	0	1.33

Fig. 4. Cumulative CO₂ transfer and hydrogen ion production during an anaerobic period of the SBR, from which the anaerobic CO₂ production was found to be 0.20 C-mol C-mol⁻¹ of propionate uptake [equation (2)].

99.8 %, while a redox balance yields 93.3 % recovery. Fig. 5 shows the oxygen uptake rate and CO₂ transfer rate for a batch test of an extended aerobic cycle using the TOGA sensor. The total oxygen consumption shown in Table 3 was obtained through numerical integration of the oxygen uptake rate for a series of three aerobic periods. The aerobic CO₂ production was calculated using equation (3) from the measured cumulative CO₂ transfer and hydrogen ion production from the TOGA sensor, as well as the ammonia uptake obtained through off-line analysis and shown in Table 3.

Due to the absence of nitrification in the SBR, the ammonia uptake could be used to calculate the biomass growth through dividing the ammonia uptake by the nitrogen coefficient from the experimentally determined biomass composition (CH_{1.89}O_{0.62}N_{0.18}). The biomass growth was estimated to be 1.96 C-mmol l⁻¹ by this method (see Table 3). Biomass growth may also be estimated using the wastage of biomass per cycle, after taking into consideration the PHA and glycogen storage products in the sludge. Using this method, the biomass growth was estimated to be 1.94 C-mmol l⁻¹. These estimations are within 2 % of each other,

Table 3. Aerobic stoichiometry of carbon, nitrogen and oxygen transformations for GAOs enriched with propionate

All results obtained were the mean of experimental data from three separate TOGA tests. The anaerobic propionate consumption was 6.1 C-mmol l⁻¹ and the biomass concentration was 65.2 C-mmol l⁻¹ for these tests.

Parameter	Aerobic stoichiometry	Units
Glycogen production	7.56	C-mmol l ⁻¹
PHB consumption	0.36	C-mmol l ⁻¹
PHV consumption	4.42	C-mmol l ⁻¹
PH2MV consumption	5.75	C-mmol l ⁻¹
Ammonium consumption	0.36	N-mmol l ⁻¹
Biomass production	1.96	C-mmol l ⁻¹
O ₂ consumption	2.64	mmol l ⁻¹
CO ₂ production	1.04	C-mmol l ⁻¹

supporting the hypothesis that either method may be used to calculate the biomass growth, which has been suggested by Zeng *et al.* (2003b).

Anaerobic and aerobic maintenance characterization

It has been hypothesized that the sole energy source for anaerobic maintenance in GAOs is obtained through glycogen hydrolysis. An equation to describe the anaerobic maintenance process by GAOs has been proposed (Filipe *et al.*, 2001a; Zeng *et al.*, 2003b):

In this study, the anaerobic batch test without VFA addition allowed the estimation of the specific anaerobic maintenance coefficient (m^{ATP}) [in mol ATP (C-mol biomass)⁻¹ h⁻¹]. The glycogen hydrolysis rate obtained from this batch test is shown in Fig. 6, and was found to be 7.0×10^{-3} C-mol (C-mol biomass)⁻¹ h⁻¹. The corresponding m^{ATP} value is therefore determined to be 3.5×10^{-3} mol ATP (C-mol biomass)⁻¹ h⁻¹ from equation (4). The PHA accumulation observed in the same anaerobic batch test is also shown in Fig. 6. The rate of PHA formation was found to be 6.2×10^{-3} C-mol (C-mol biomass)⁻¹ h⁻¹, where the composition of PHA produced was calculated as 12 % PHB, 41 % PHV and 47 % PH2MV. The rate of CO₂ formation was 2.1×10^{-3} C-mol (C-mol biomass)⁻¹ h⁻¹ from equation (2) using the TOGA data (not shown). The results obtained in this study are not well described by the proposed stoichiometry for anaerobic maintenance processes in equation (4). The reason for this is not well understood. Perhaps when the wastewater contains no VFA the maintenance process of GAOs is different to that when VFAs are present, or the maintenance of acetate-enriched GAOs (often dominated by *Competibacter*) may be different from that of these Alphaproteobacteria GAOs enriched on propionate. It should also be noted that equation (4) has not

Fig. 5. Oxygen uptake rate and CO₂ transfer rate from an extended aerobic period for determination of the energy required for aerobic cell maintenance.

yet been validated experimentally, and may not describe the actual anaerobic maintenance process of GAOs.

The batch test performed with an extended aerobic period to estimate the aerobic maintenance process is shown in Fig. 5. After 4 h aeration, the rate of oxygen consumption (m_{os}) [in mol O₂ (C-mol biomass)⁻¹ h⁻¹] became relatively constant at a mean value of 0.22 mmol h⁻¹, or 2.7×10^{-3} mol (C-mol biomass)⁻¹ h⁻¹. The oxygen uptake rate correlated well with the CO₂ transfer rate during this time period (see Fig. 5), suggesting that almost all of the oxygen was converted to CO₂, which is consistent with previous studies under maintenance conditions (Zeng *et al.*, 2003b). The anaerobic and aerobic maintenance coefficients are compared with literature-reported values from previous studies with PAOs and GAOs in Table 4. It may be observed that the maintenance energy requirements are similar between PAOs and GAOs, while the m^{ATP} values are slightly higher in both PAO and GAO cultures enriched with propionate as compared to acetate.

Impact on the competition between PAOs and GAOs

Recent studies have suggested that a propionate feed source is more beneficial for reliable EBPR performance than acetate (Chen *et al.*, 2004; Oehmen *et al.*, 2005a, 2006; Pijuan *et al.*, 2004; Thomas *et al.*, 2003), likely due to a reduced level of GAO activity. The metabolic activity of PAOs and GAOs fed with different carbon sources is compared in Table 4. The maximum specific VFA uptake rate (q^{max}) [in C-mol (C-mol biomass)⁻¹ h⁻¹] and the aerobic biomass growth yield (Y_{growth}) [in C-mol biomass growth (C-mol VFA uptake)⁻¹] are factors that may influence the competition between PAOs and GAOs in wastewater systems. The q^{max} and Y_{growth} results from this study were very similar to most of the previously reported values for PAOs and GAOs, which does not clearly suggest that PAOs possess an advantage over GAOs when propionate is the carbon source. The reason for the observed advantage of PAOs in previous studies in which propionate was the carbon source is currently unknown.

Fig. 6. The rate of glycogen hydrolysis and PHA accumulation observed during an anaerobic batch test without propionate addition. The energy required for anaerobic maintenance was calculated to be 3.5×10^{-3} mol ATP (C-mol biomass)⁻¹ h⁻¹ from equation (4).

Table 4. Comparison of VFA uptake rate, biomass growth yield and maintenance coefficients between this study and other PAO and GAO studies

NA, Not available.

Carbon source	Organism	Publication	$q^{\max}$	Y_{growth}^*	m^{ATP}	m_{os}^*
Propionate	GAO	This study	0.18	0.32	3.5×10^{-3}	2.7×10^{-3}
Propionate	PAO	Oehmen <i>et al.</i> (2005b)	0.18	0.35	4.2×10^{-3}	3.8×10^{-3}
Acetate	GAO	Zeng <i>et al.</i> (2003b)	0.17	0.37	2.4×10^{-3}	3.5×10^{-3}
Acetate	GAO	Filipe <i>et al.</i> (2001a)	0.24	NA	2.1×10^{-3}	NA
Acetate	PAO	Smolders <i>et al.</i> (1995)	0.43	0.34	2.5×10^{-3}	4.5×10^{-3}
Acetate	PAO	Filipe <i>et al.</i> (2001b)	0.19	NA	2.0×10^{-3}	NA

* Y_{growth} and m_{os} were not presented previously in Oehmen *et al.* (2005b), although these parameters were obtained during that study.

Further investigation is required to assess the proliferation of these Alphaproteobacteria GAOs in EBPR plants as well as their ability to compete with PAOs in these systems.

The presence of these alphaproteobacteria GAOs in EBPR systems could have considerable ramifications for future investigations concerning the competition between PAOs and GAOs. *Accumulibacter* appears to be a dominant PAO in the presence of both acetate and propionate as carbon sources, and has been shown to have the capacity to switch between acetate and propionate uptake without the need for acclimation (Oehmen *et al.*, 2005a; Pijuan *et al.*, 2004). It is possible, however, that GAOs have a more specific preference for acetate or propionate. In one study, GAOs enriched with acetate as the sole carbon source (and dominated by *Competibacter*) were shown to be far less effective in propionate uptake, while GAOs enriched with propionate (dominated by Alphaproteobacteria) were shown to take up acetate at less than half the rate of propionate (Oehmen *et al.*, 2005a). A carbon source preference exhibited by different groups of GAOs could perhaps be useful in minimizing the undesirable proliferation of GAOs in EBPR systems.

Conclusions

The anaerobic and aerobic metabolism of GAOs enriched with propionate as the sole carbon source has been described in this study. The main outcomes from this study can be summarized as follows.

The proposed metabolic model described well the anaerobic biochemical transformations by GAOs with propionate as the carbon source.

The biomass was highly enriched in Alphaproteobacteria with a tetrad morphotype. A fraction of these organisms were identified as *D. vanus*-related organisms. Furthermore, most Alphaproteobacteria in the biomass exhibited intracellular anaerobic-aerobic cycling of PHA.

The maximum specific VFA uptake rate and biomass growth yield of these Alphaproteobacteria GAOs were similar to

values reported in the literature from PAO and GAO studies. It is unclear at present why these organisms seem to compete less effectively than PAOs when propionate is the carbon source, as has been suggested by previous studies.

The energy requirements for anaerobic and aerobic maintenance processes found in this study were similar to previously reported values for PAOs and GAOs.

Currently available oligonucleotide probes for GAO bound only 16 % of the Bacteria in this system, whereas many more appeared to be cycling PHA, suggesting the presence of other GAOs. Further investigation into the phylogenetic diversity of GAOs, and their significance in EBPR systems, is recommended.

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