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ORIGINAL ARTICLE

Anaerobic glyoxylate cycle activity during simultaneous utilization of glycogen and acetate in uncultured *Accumulibacter* enriched in enhanced biological phosphorus removal communities

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Enhanced biological phosphorus removal (EBPR) communities protect waterways from nutrient pollution and enrich microorganisms capable of assimilating acetate as polyhydroxyalkanoate (PHA) under anaerobic conditions. *Accumulibacter*, an important uncultured polyphosphate-accumulating organism (PAO) enriched in EBPR, was investigated to determine the central metabolic pathways responsible for producing PHA. Acetate uptake and assimilation to PHA in *Accumulibacter* was confirmed using fluorescence *in situ* hybridization (FISH)-microautoradiography and post-FISH chemical staining. Assays performed with enrichments of *Accumulibacter* using an inhibitor of glyceraldehyde-3-phosphate dehydrogenase inferred anaerobic glycolysis activity. Significant decrease in anaerobic acetate uptake and PHA production rates were observed using inhibitors targeting enzymes within the glyoxylate cycle. Bioinformatic analysis confirmed the presence of genes unique to the glyoxylate cycle (isocitrate lyase and malate synthase) and gene expression analysis of isocitrate lyase demonstrated that the glyoxylate cycle is likely involved in PHA production. Reduced anaerobic acetate uptake and PHA production was observed after inhibition of succinate dehydrogenase and upregulation of a succinate dehydrogenase gene suggested anaerobic activity. Cytochrome *b/b*$_6$ activity inferred that succinate dehydrogenase activity in the absence of external electron acceptors may be facilitated by a novel cytochrome *b/b*$_6$ fusion protein complex that pushes electrons uphill to more electronegative electron carriers. Identification of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase genes in *Accumulibacter* demonstrated the potential for interconversion of C_3 intermediates of glycolysis and C_4 intermediates of the glyoxylate cycle. Our findings along with previous hypotheses from analysis of microbiome data and metabolic models for PAOs were used to develop a model for anaerobic carbon metabolism in *Accumulibacter*.
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Introduction

Enhanced biological phosphorus removal (EBPR) is a wastewater treatment process that protects receiving waters from eutrophication by enriching microorganisms capable of storing volatile fatty acids (VFAs) as polyhydroxyalkanoates (PHA) under anaerobic conditions (no external electron acceptors) in the first phase of the process. A distinct physiological group of microorganisms enriched in EBPR communities, known as polyphosphate-accumulating organisms (PAOs), anaerobically uptake VFAs and store them as PHA. In one PAO, known as *Accumulibacter*, acetate uptake is driven by active transport processes utilizing the electrochemical gradient of the proton-motive force (PMF). Generation of the PMF is hypothesized to occur via efflux of phosphate (P_i) (derived from polyphosphate (polyP) breakdown) in symport with protons across

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the cell membrane (Saunders *et al.*, 2007; Burow *et al.*, 2008). Reducing equivalents for PHA formation are generated by simultaneously catabolizing intracellular glycogen. However, some investigations have demonstrated that the budget for reducing power cannot be met exclusively by glycogen catabolism and anaerobic carbon (C) flux through the tricarboxylic acid (TCA) or glyoxylate cycles may provide this shortfall (Pereira *et al.*, 1996; Pramanik *et al.*, 1999; Lemos *et al.*, 2003; Schuler and Jenkins, 2003). During the subsequent aerobic phase of EBPR, PAOs grow and reproduce using intracellular PHA and the TCA cycle. PAOs also uptake inorganic P_i and convert it to intracellular polyP during this phase. The PAOs are then gravity settled and separated from the wastewater, allowing low phosphorus (P) wastewater to be discharged to the environment (Seviour *et al.*, 2003).

No PAOs are available in pure culture (Seviour *et al.*, 2003; Oehmen *et al.*, 2007), thus anaerobic central metabolic pathway models that describe C flux in these microorganisms have been developed with enrichment cultures. These models have been limited by non-existent or insufficient microbial community structure knowledge (Pereira *et al.*, 1996; Hesselmann *et al.*, 2000; Louie *et al.*, 2000; Filipe *et al.*, 2001; Lemos *et al.*, 2003) or a lack of evidence for their physiological function (Garcia Martin *et al.*, 2006). *In situ* structure-function studies of *Accumulibacter* (Lee *et al.*, 2003; Kong *et al.*, 2004) have linked phylogeny to function using fluorescence *in situ* hybridization-microautoradiography (FISH-MAR) and post-FISH chemical staining to clarify some aspects of PAO physiology.

To provide a more detailed model of metabolic pathways involved in anaerobic carbon metabolism in *Accumulibacter*, we carried out *in situ* structure-function analyses and enzyme inhibition investigations combined with biochemical process data acquisition. Bioinformatic analysis of the *Accumulibacter* genome and measurement of mRNA transcripts using relative quantitative reverse-transcriptase PCR provided further insight into the expression of genes in these pathways.

Materials and methods

Accumulibacter *enrichment culture*

Accumulibacter were highly enriched (see 'Results') from a seed sludge taken from a local full-scale EBPR wastewater treatment plant (Queensland, Australia) in an 8 l laboratory-scale bioreactor that exhibited good P_i removal. Detail of the bioreactor's operation has been previously reported (Lu *et al.*, 2006).

Acetate uptake assays

Biomass was harvested from a parent bioreactor at the end of the aerobic period and resuspended in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered salts medium (pH 7.5) containing trace metals at a final concentration of 250 μg ml^{-1} of total protein. Protein concentration was determined using the BCA protein assay kit using bovine serum albumin as a standard (Pierce Biotechnology, Rockford, IL, USA). The HEPES-buffered salts medium and acetate uptake assays have been previously described (Burow *et al.*, 2008). Briefly, anaerobic uptake assays were carried out in triplicate in 100 ml batch tests in conical flasks that used oxygen-free nitrogen or helium sparging (15 min) to obtain anaerobic conditions. Batch tests were initiated by the addition of 2 mM acetate and samples were taken at T = 1, 10, 20 and 30 min, with uptake rates calculated and subjected to statistical analysis using the Student's *t*-test. To elucidate anaerobic activity of central metabolic pathways, cells were treated with inhibitors 20 min prior to the addition of acetate. Iodoacetate was used to target glyceraldehyde-3-phosphate dehydrogenase, an enzyme in glycolysis; (mono)fluoroacetate-targeted aconitase, an enzyme common to the glyoxylate and TCA cycles; 3-nitropropionate and itaconate-targeted isocitrate lyase, an enzyme unique to the glyoxylate cycle; malonate-targeted succinate dehydrogenase, an enzyme that oxidizes succinate to fumarate and oxantel-targeted fumarate reductase, an enzyme that reduces fumarate to succinate (see Table 1 for concentrations used).

Anaerobic:aerobic batch tests were carried out with 200 ml of enriched *Accumulibacter* biomass harvested from the parent bioreactor at the end of the aerobic phase. The 200 ml subsample was sparged for 15 min with oxygen-free helium to ensure anaerobic conditions. Acetate (2 mM) was then added and biomass incubated under oxygen-free conditions for 60 min, which induced intracellular PHA storage. Acetate was measured at the end of the 60 min period to verify its complete consumption. PHA stored under anaerobic conditions was the sole carbon and energy source used by the *Accumulibacter* enrichment culture in the subsequent aerobic phase. Aerobic conditions were applied to the batch test by sparging with air. PHA was measured at the end of both the anaerobic phase (60 min anaerobic incubation) and the aerobic phase (120 min aerobic incubation) in the enrichment culture.

Chemical analyses

Biomass samples taken during uptake assays for liquid phase (extracellular) analysis were filtered using a sterile 0.22 μm filter (Millipore, Billerica, MA, USA) and stored at 4 or −20 °C for subsequent chemical analyses. Acetate was analysed by high-pressure liquid chromatography (HPLC; Bio-Rad, Hercules, CA, USA) and P_i was determined using a flow injection analyzer (FIA, Lachat Instruments, Loveland, CO, USA). Samples taken for solid phase (intracellular) analysis of glycogen and PHA were fixed with formaldehyde. Glycogen was measured

Table 1 Effect of inhibitors on anaerobic acetate uptake, P_i release and PHA production in *Accumulibacter*

Treatment	*Target*	*Acetate uptake rate (nmol min^{-1} mg^{-1} protein)*[a]		*P_i release rate (nmol min^{-1} mg^{-1} protein)*		*PHA production (C-mmol g^{-1} dry cell weight)*	
		No inhibitor	*Inhibitor*	*No inhibitor*	*Inhibitor*	*No inhibitor*	*Inhibitor*
Fluoroacetate (0.02 mM)	Aconitase	153 ± 11	42 ± 2 (**56**)[b]	—[c]	—	—	—
Fluoroacetate (0.2 mM)	Aconitase	136 ± 9	42 ± 2 (**69**)	—	—	—	—
Fluoroacetate (1 mM)	Aconitase	136 ± 9	34 ± 9 (**75**)	151 ± 8	48 ± 7 (**68**)	4.3 ± 0.4	1.4 ± 0.2 (**67**)
3-Nitropropionate (0.2 mM)	Isocitrate lyase	192 ± 10	96 ± 13 (**50**)	—	—	—	—
3-Nitropropionate (1 mM)	Isocitrate lyase	192 ± 10	61 ± 4 (**68**)	201 ± 16	56 ± 2 (**72**)	—	—
3-Nitropropionate (5 mM)	Isocitrate lyase	192 ± 10	41 ± 5 (**79**)	—	—	—	—
Itaconate (10 mM)	Isocitrate lyase	217 ± 3	63 ± 12 (**71**)	260 ± 11	77 ± 7 (**71**)	4.9 ± 0.2	1.5 ± 0.1 (**69**)
Malonate (10 mM)	Succinate dehydrogenase	217 ± 3	141 ± 10 (**35**)	260 ± 11	148 ± 23 (**43**)	4.9 ± 0.2	3.4 ± 0.3 (**30**)
Oxantel (1 mM)[d]	Fumarate reductase	179 ± 8	181 ± 10 (0)	182 ± 7	181 ± 7 (1)	4.7 ± 0.3	4.6 ± 0.3 (3)
Average	NA[e]	175 ± 30	NA	199 ± 43	NA	4.6 ± 0.3	NA

Abbreviation: PHA, polyhydroxyalkanoate.
[a]Average acetate uptake rates were determined from assays performed in triplicate and carried out with 2 mM acetate in HEPES-buffered medium in the absence or presence of various inhibitors, see 'Materials and methods' for full details.
[b]The values in parentheses are percentages of reduction in acetate uptake rates, P_i release rates and PHA production. Reduction of acetate uptake rates, P_i release rates or PHA production was considered significant if the P-value < 0.05 and is denoted by bold text.
[c]Not determined.
[d]Determination of anaerobic acetate uptake rates and P_i release rates in the presence of oxantel was previously reported (Burow *et al.*, 2008). All assays performed with oxantel were carried out at pH 8.
[e]Not applicable.

by lyophilization and digestion of biomass with HCl and the resulting supernatant liquid obtained after HCl digestion was analysed for glucose by HPLC (Bond *et al.*, 1999). PHA analysis was determined on lyophilized biomass, acidified in a methanol solution using gas chromatography (Oehmen *et al.*, 2005).

Fluorescence in situ *hybridization and quantitative FISH*

FISH was carried out with FISH probes listed in Supplementary Tables S1–S3 on fixed samples of biomass as previously described (Amann, 1995). Digital image analysis (ImageJ V1.35k; http://rsb.info.nih.gov/ij/) was used for quantitative FISH with biomass hybridized with Cy3-labelled specific probes and Cy5-labelled EUBMIX probes by calculating the percentage area of the specific probe fluorescence relative to the EUBMIX fluorescence (Burow *et al.*, 2007).

FISH-MAR and post-FISH chemical staining

Combined FISH and MAR was carried out as described in Lee *et al.* (1999) on biomass samples enriched in *Accumulibacter* using PAOMIX and EUBMIX probes and radiolabelled acetate (^{14}C) to determine uptake of acetate in these microorganisms in the absence of external electron acceptors (anaerobic conditions). A mixture of acetate (final concentration 2 mM) including radiolabelled acetate (specific activity 2.15 GBq mmol^{-1}; final radioactive concentration 37 kBq ml^{-1}) and unlabelled acetate was incubated with biomass for 2 h under anaerobic conditions prior to FISH-MAR. Assimilation of acetate and conversion to PHA was determined in *Accumulibacter* by post-FISH chemical staining using PAOMIX and EUBMIX probes and Nile Blue A staining according to previously described methods (Ostle and Holt, 1982; Crocetti *et al.*, 2000).

RNA stabilization, extraction and cDNA synthesis

Expressed genes (mRNA transcripts) were measured in *Accumulibacter* using relative quantitative reverse-transcriptase PCR (qRT-PCR). The mRNA transcripts in 2 ml (250 μg protein ml^{-1}) of biomass samples were stabilized by incubation with 4 ml of a 5% (v/v) phenol, 95% (v/v) ethanol solution according to methods recommended by the Institute for Food Research Microarray Facility (http://www.ifr.ac.uk). Samples were centrifuged at 21 000 *g* for 10 min at 4 °C and pellets were retained for storage at −80 °C.

Stabilized biomass pellets were thawed on ice and resuspended in 300 μl of RLT buffer (containing guanidine thiocyanate; RNeasy mini kit; Qiagen, Valencia, CA, USA). Floccular biomass was then dispersed by repeated pipetting through increasingly smaller pipette tips. Lysozyme was added to biomass at a final concentration of 2 mg ml^{-1}, mixed

by pipetting and incubated for 5 min at 37 °C with intermittent vortexing. Centrifugation was carried out at 21 000 *g* for 1 min and the resulting supernatant was transferred to a fresh tube. The manufacturer's protocol for bacterial RNA extraction was followed from this stage (RNeasy mini kit; Qiagen) except that the resulting RNA was eluted in 30 μl of RNase-free H_2O containing 1 μl of RNase inhibitor (RNaseOut; Invitrogen). DNase treatment of RNA extracts using Turbo DNase (Ambion, Austin, TX, USA) was performed according to the manufacturer's protocol. RNase inhibitor (1 μl) (RNaseOut; Invitrogen) was added to the DNA-free RNA and stored at −80 °C.

RNA quality and quantity was checked by gel electrophoresis and spectrophotometric analysis (ND-1000; NanoDrop Technologies) prior to synthesis of cDNA templates. Approximately 2 μg of high-quality total RNA was reverse transcribed using the SuperScript III reverse transcriptase kit (Invitrogen) as per the manufacturer's protocol.

Primer design and qRT-PCR analysis

Primers targeting *Accumulibacter* metabolic genes were designed using nucleotide sequence data from strains of sequenced *Accumulibacter* (Garcia Martin *et al.*, 2006). Preliminary sequence data from *Accumulibacter* and other bacteria was obtained from the integrated microbial genomes with microbiome samples (IMG/M) database (http://img.jgi.doe.gov/m). Metabolic gene databases containing 30–40 genes each were constructed for aconitase B (*acnB*), isocitrate lyase (*ICL*), succinate dehydrogenase B (*sdhB*) and cytochrome b/b_6 (cyb/b_6). The sequences were aligned with ClustalW (Thompson *et al.*, 1994) and imported into ARB for further examination (Ludwig *et al.*, 2004). Nucleotide regions conserved among strains of *Accumulibacter* but with mismatches to other bacteria were targeted for primer design. Primers were designed using Primer Express V1.5 (Applied Biosystems, Foster City, CA, USA) for amplification and detection of PCR products using SYBR Green chemistry.

Primer sets were evaluated after PCR amplification on the Real-Time ABI Prism 7000 detection system (Applied Biosystems) by gel electrophoresis and sequencing on 3730xl DNA Analyzer (Applied Biosystems). Each PCR volume was 25 μl and included 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), 5 μl primer (final concentration, 100–400 nM), 5 μl cDNA template and 2.5 μl high-purity pyrogen-free (clinical grade) water. Negative control PCRs included no template controls and RNA-only controls (RNA without reverse-transcriptase cDNA synthesis performed) to check for the presence of contaminating DNA. The thermal cycling parameters included an Ampli*Taq* Gold (Applied Biosystems) activation step at 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Primer sets confirmed to amplify genes belonging to *Accumulibacter* are listed in Table 2.

Gene expression of target (metabolic) genes was determined relative to a reference or housekeeping gene (16S rRNA) in three separate anaerobic:aerobic batch tests and the fold-relative gene expression was calculated by the method described by Pfaffl (2001). All primer sets used in qRT-PCR were developed in this study except the 16S rRNA primer set, which was previously reported by Saunders (2005).

Results

Microbial community structure of biomass used in acetate uptake assays

Biomass samples from a bioreactor demonstrating efficient and stable P-removal (reduced P_i from 20–40 mg P_i per litre to <0. 2 mg P_i or 0.645–1.29 mM to <6 μM) were harvested for use in acetate uptake assays. Further details of the process performance of this bioreactor is detailed elsewhere (Lu *et al.*, 2006).

The microbial community structure in subsamples taken from the parent bioreactor were similar (although not identical) to those reported by Burow *et al.* (2008), as biomass was sampled from the same

Table 2 Primers used for qRT-PCR analysis of *Accumulibacter*

Gene	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*Primer-binding region*[a]	*Amplicon size (bp)*
16S rRNA	CCTTTTAGTGCCGTAGCTAACG	GGATTCCTGACATGTCAAGGGT	F810–831; R951–972 (2000470820)	163
Aconitase B (*acnB*)	GATGCTCGGTGGCTACAACG	CTTGACATCGTGGAAGAAGTCG	F66–85; R165–186 (2000381700)	121
Isocitrate lyase (*ICL*)	AAGGTCGTCGAGCGCATC	CATCAGTTCGAAGGCGTTGAG	F349–366; R490–510 (2000428210)	162
Succinate dehydrogenase (*sdhB*)	ATCCGGACAAGTTCGTCGG	GGACAGACATCGACGCAATTC	F491–509; R627–647 (2000392840)	157
Cytochrome b/b_6 (cyb/b_6)	ATGCTGCTGCACCTCGTTC	CAGTTGGTCCCAGACGATCC	F274–292; R395–414 (2000461790)	141

[a]Nucleotide positions of genes targeted by primers in *Accumulibacter* (F, forward primer; R, reverse primer) according to IMG/M (http://img.jgi.doe.gov/m); gene object ID in parenthesis.

bioreactor over a similar time period during March–October 2006. *Accumulibacter* were the dominant microorganism across all biomass subsamples and ranged from 79(±5) to 85(±2)% of *Bacteria* (Supplementary Table S4). The range is statistically insignificant ($P = 0.12$), providing evidence that the community structure was stable across each biomass sample used in acetate uptake assays.

Although biomass sampled from the bioreactor consisted of a mixed microbial community, conclusions from the use of metabolic inhibitors were made in regards to *Accumulibacter* central metabolism. This was possible as they were the dominant microorganism within the sludge community and because this microorganism is likely to be responsible for the majority of carbon transformations observed in these uptake assays. Results from FISH-MAR, post-FISH chemical staining and qRT-PCR assays are specific to *Accumulibacter*. FISH-MAR and post-FISH chemical staining showed that comparatively very few other microorganisms (<5%) were assimilating acetate and/or producing PHA.

Anaerobic acetate uptake, P_i release and PHA production in the Accumulibacter *enrichment*

Anaerobic assays were performed using whole cells sampled at the end of the aerobic phase from the parent bioreactor. Radiolabelled acetate was taken up by *Accumulibacter* as determined by FISH-MAR (Supplementary Figure S1) and assimilated to PHA as determined by post-FISH staining with Nile Blue A (Supplementary Figure S2). The *Accumulibacter* enrichment took up acetate (2 mM) at an average rate of 175 ± 30 nmol min^{-1} mg^{-1} protein (Table 1). Anaerobic acetate uptake was linear over the initial 30 min (Supplementary Figure S3). Acetate was assimilated by the *Accumulibacter* enrichment as PHA with monomer ratios of hydroxybutyrate (HB) to hydroxyvalerate (HV) of 90:10.

Acetate uptake stimulated a concomitant P_i release rate for the *Accumulibacter* enrichment of 199 ± 43 nmol min^{-1} mg^{-1} protein (Table 1) and remained linear over the initial 30 min (Supplementary Figure S4). The ratio between the average P_i release rate to average acetate uptake rate across control batch tests ($n = 12$) was 0.57 P-mol C-mol^{-1}. This ratio supported the finding that the sampled microbial community was highly enriched in *Accumulibacter* possessing the PAO phenotype (McMahon *et al.*, 2002; Lu *et al.*, 2006).

Anaerobic carbohydrate metabolism in the Accumulibacter *enrichment*

Models of carbon metabolism in PAOs predict that glycogen catabolism is an important source of energy (ATP) and reducing power ($NADH_2$) under anaerobic conditions (Mino *et al.*, 1998; Oehmen *et al.*, 2007). Consumption of glycogen (stored glucose) was observed in the *Accumulibacter* enrichment during 60 min incubation with 2 mM acetate under anaerobic conditions. Glycogen decreased from 3.3 ± 0.2 C-mmol glucose per g dry cell weight at the beginning of the incubation period to 1.9 ± 0.13 C-mmol glucose g^{-1} dry cell weight at the end of the period. The effect of inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; key enzyme in glycolysis) on glycogen catabolism was investigated by adding 1 mM iodoacetate (Bickis and Quastel, 1965; Even *et al.*, 1999) to an anaerobic incubation prior to the addition of 2 mM acetate. After 60 min incubation with acetate under inhibited conditions the intracellular level of glycogen (3.0 ± 0.3 C-mmol glucose g^{-1} dry cell weight) did not decrease and was at the same level measured at the beginning of the incubation. This suggested that glucose generated from glycogen is catabolized via the glycolytic pathway.

Anaerobic assimilation of acetate as PHA and tricarboxylic acid metabolism in the Accumulibacter *enrichment*

It is currently accepted that glycogen catabolism does not provide all the reducing power for generation of PHA from acetate in PAOs (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). According to one metabolic model, PAOs generate approximately 30% of the overall reducing equivalents for anaerobic PHA production from C flux through the full oxidative TCA cycle (Pereira *et al.*, 1996). Other metabolic models have proposed C flux through the glyoxylate cycle (Yagci *et al.*, 2003) or the reductive and oxidative branches of the TCA pathways (split TCA cycle) (Pramanik *et al.*, 1999).

Acetate uptake and production of PHA was investigated in the presence of the aconitase inhibitor, fluoroacetate (Lotspeich *et al.*, 1952). The first step of the TCA and glyoxylate cycles is catalysed by citrate synthase, which converts fluoroacetate to an inhibitory fluorocitrate compound that tightly binds aconitase (Lauble *et al.*, 1996) blocking C flow through the TCA or glyoxylate cycles. The acetate uptake rate of the *Accumulibacter* enrichment was strongly inhibited in the presence of fluoroacetate compared with the solvent-only (uninhibited) control. Fluoroacetate similarly inhibited concomitant P_i release rates and PHA production compared to controls (Table 1; Supplementary Figures S3 and S4).

The effect of 3-nitropropionate and itaconate, compounds known to inhibit ICL an enzyme unique to the glyoxylate cycle (Hillier and Charnetzky, 1981; Bentrup *et al.*, 1999; Munoz-Elias and McKinney, 2005), was examined in the *Accumulibacter* enrichment. 3-Nitropropionate substantially inhibited acetate uptake and concomitant P_i release rates (Table 1; Supplementary Figures S5 and S6). The acetate uptake rate, P_i release rate and PHA

production in the presence of itaconate were all substantially inhibited in the *Accumulibacter* enrichment (Table 1; Supplementary Figure S7).

Activity of succinate dehydrogenase in *Accumulibacter* has been predicted even in the absence of external electron acceptors due to the presence of a novel fusion protein comprising a cyb/b_6 complex. It is theorized that reverse electron transport through cyb/b_6 driven by the PMF (Δp) could reoxidize reduced quinones produced by succinate dehydrogenase under anaerobic conditions (Garcia Martin *et al.*, 2006). It has been previously shown that acetate uptake in *Accumulibacter* is sensitive to uncoupling of the PMF using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or selective inhibition of the electrical potential ($\Delta\psi$) by incubation with valinomycin (Burow *et al.*, 2008). Anaerobic PHA production in the *Accumulibacter* enrichment after 60 min incubation with 2 mM acetate resulted in a substantial decrease in PHA production down from 4.25 ± 0.39 C-mmol g^{-1} dry cell weight in the control incubation to 0.97 ± 0.17 C-mmol g^{-1} dry cell weight in the CCCP (final concentration 100 μM) inhibited incubation. In the presence of malonate, a reversible competitive inhibitor of succinate dehydrogenase (Thorn, 1953; Sumegi *et al.*, 1990), acetate uptake, P_i release and PHA production were all significantly inhibited (Table 1; Supplementary Figure S8).

Genes encoding fumarate reductase have been identified in *Accumulibacter* (Garcia Martin *et al.*, 2006). However, anaerobic PHA production in the *Accumulibacter* enrichment after 60 min incubation with 2 mM acetate resulted in a similar level of PHA production in control- and oxantel-inhibited incubations inferring a lack of fumarate reductase activity under these conditions (Table 1). This is consistent with a previous investigation that demonstrated no effect of oxantel on acetate uptake or P_i release rates in *Accumulibacter* (Burow *et al.*, 2008).

Bioinformatic analysis of sequenced strains of Accumulibacter

Glycolysis activity in *Accumulibacter* has been previously suggested based exclusively on sequence data analysis that reported the presence of genes in the Embden–Meyerhof–Parnas (EMP) pathway of glucose metabolism and the absence of genes in the Entner–Doudoroff (ED) pathway of glucose metabolism (Garcia Martin *et al.*, 2006). Glucose metabolism via the pentose phosphate (PP) pathway has also been suggested in models of anaerobic PAO metabolism (Pramanik *et al.*, 1999). Investigation of *Accumulibacter* sequence data deposited in the IMG/M database was carried out to identify potential activity of the PP pathway. Glucose 6-P dehydrogenase gene (*zwf*), a gene unique to the PP pathway, was absent in sequenced strains of *Accumulibacter*.

Results from inhibition of glyoxylate cycle activity in the *Accumulibacter* enrichment prompted a search for genes in this cycle in the *Accumulibacter* metagenome data set. Genes unique to the glyoxylate cycle (*ICL* and malate synthase, *MS*) were identified in *Accumulibacter* (both US and OZ strains) in this study (Supplementary Figure S9).

It has been previously reported that the malic enzyme (*malE*) gene encoding an enzyme capable of interconverting C_3 intermediates of the glycolytic pathway and C_4 intermediates of the glyoxylate cycle (or TCA cycle) is present in *Accumulibacter* (Garcia Martin *et al.*, 2006). Other genes that similarly catalyse C_3:C_4 interconversions (phosphoenolpyruvate (PEP) carboxylase, *ppc* and PEP carboxykinase, *pck*) were identified in sequenced strains of *Accumulibacter* in this study (Supplementary Figure S9).

Expression of tricarboxylic acid pathway genes under anaerobic:aerobic conditions in Accumulibacter

To provide molecular evidence of C flux and electron transport through central metabolic pathways in *Accumulibacter*, mRNA transcripts of metabolic genes were quantified relative to 16S rRNA gene transcripts using qRT-PCR. The 16S rRNA gene and metabolic genes amplified with primers listed in Table 2 using qRT-PCR were of the expected size (Supplementary Figure S10) and had greater sequence identity with the corresponding genes of sequenced strains of *Accumulibacter* than any other microorganisms (Table 3).

The biomass samples used in assays for subsequent qRT-PCR analysis were taken at the end of the aerobic phase from the bioreactor enriched in *Accumulibacter* and subjected to a complete anaerobic:aerobic EBPR cycle in batch tests. Acetate (2 mM) was provided in the absence of external electron acceptors and PHA was produced (4.75 ± 0.42 C-mmol g^{-1} dry cell weight) after 60 min anaerobic incubation. Under subsequent aerobic conditions, PHA was depleted (0.25 ± 0.04 C-mmol g^{-1} dry cell weight) after 120 min incubation. Biomass was sampled from batch tests under anaerobic conditions prior to the addition of acetate to obtain a baseline transcript profile. The anaerobic transcriptional response to the addition of acetate was measured after 10 and 30 min of anaerobic incubation with acetate. The aerobic transcriptional response was measured after 10 and 30 min of aeration subsequent to the completion of a 60 min anaerobic incubation with acetate.

The expression of glyoxylate cycle genes, *acnB* and *ICL*, increased under anaerobic conditions in the presence of acetate. Expression of these genes was further enhanced under subsequent aerobic conditions compared to baseline anaerobic conditions where no C was available. The transcript profile data showed that *acnB* increased 2.3-fold after 10 min and 3.6-fold after 30 min under anaerobic conditions (Figure 1a) and further increased (5.2-fold after 10 min and 4.9-fold respectively after

Table 3 Results of nucleotide sequencing of amplicons and BLASTN searches of the nucleotide sequences

Target	*Amplicon sequence*[a]	*Closest sequences in databases*[b]	*Identities*[c]	*E-value*[d]
16S rRNA	**CCTTTTAGTGCCGTAGCTAACG**CGTGAAGTTGACCGCCTGGG GAGTACGGCCGCTAGGCTAAAACTCTAAGGAATTGACGGGGA CCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGC GAAAAACCTTACCT**ACCCTTGACATGTCAGGAATCC**	*Accumulibacter phosphatis*; 16S rRNA (Gene object ID 2000470820)	161/163 (98%)	1e−81
		Clone LV2_H10; 16S rRNA (Accession number EF565161)	161/163 (98%)	2e−74
		Uncultured *Rhodocyclaceae*; 16S rRNA (Accession number AM268356)	160/163 (98%)	7e−74
Aconitase B (*acnB*)	**GATGCTCGGTGGCTACAACG**TCAAGCCGCTGATCGACCTCCT CAGCGGCCCGACCGGCGCCATCGCCGCCGAGGCCCTCAAGAA AACCCTGCTGGTCTT**CGACTTCTTCCACGATGTCAAG**	*Accumulibacter phosphatis*; *acnB* (Gene object ID 2000381700)	109/121 (90%)	6e−33
		Magnetospirillum magneticum AMB-1; *acnB* (Accession number AP00725AP007255)	99/123 (80%)	2e−22
		Methylibium petroleiphilum PM1; *acnB* (Accession number CP000555)	97/120 (80%)	7e−21
Isocitrate lyase (*ICL*)	**AAGGTCGTCGAGCGCATC**AACAACTCCTTCCGTCGTGCCGA TGAAATCCAGTGCTCGAAAGACATCGGTCCNGGTGACAAGG GCTACATCGACTATTTCGCGCCAATCGTCGCCGATGCCGAGG CCGGGTTTGGCGGTGTG**CTCAACGCCTTCGAACTGATG**	*Accumulibacter phosphatis*; *ICL* (Gene object ID 2000428210)	142/162 (87%)	1e−38
		Polaromonas naphthalenivorans CJ2; *ICL* (Accession number CP000529)	133/162 (82%)	2e−37
		Azoarcus sp. EbN1; *ICL* (Accession number CR555306)	130/160 (81%)	2e−35
Succinate dehydrogenase (*sdhB*)	**ATCCGGACAAGTTCGTCGG**TCCTGCCGGCCTGCTCAACGCAT ACCGCTTCATCGCCGATACCCGTGACGAGGCGACCAACGAGC GCCTGGACGACCTTGAGGATCCCTATCGACTGTTCCGCTGCCA CTCGATCAT**GAATTGCGTCGATGTCTGTCC**	*Accumulibacter phosphatis*; *sdhB* (Gene object ID 2000392840)	135/157 (85%)	5e−30
		Chromobacterium violaceum ATCC 12472; *sdhB* (Accession number AE016825)	131/155 (84%)	3e−40
		Ralstonia solanacearum GMI1000; *sdhB* (Accession number AM260479)	130/155 (83%)	1e−38
Cytochrome b/b_6 (cyb/b_6)	**ATGCTGCTGCACCTCGTTC**GCGAGTGGTCGTATGGCCGCTA TTACGGCTTCCGCCTCTATTCGTGGATGACCGGCATCCCGCT GATCTGGCTGGCGTATATCGCCGGAATCGGCGGCTACT**GGA TCGTCTGGGACCAACTG**	*Accumulibacter phosphatis*; cyb/b_6 (Gene object ID 2000461790)	126/141 (89%)	1e−37
		Mesorhizobium sp. BNC1 Plasmid 1; cyb/b_6-like (Accession number CP000389)	30/45 (66%)	6e−15
		Magnetospirillum magneticum AMB-1; *cyb* (Accession number AP007255)	28/47 (59%)	3e−16

[a]Bold text denotes the primer-binding region of the amplicon; underlined text denotes mismatches between the amplicon and the *Accumulibacter* genome.
[b]Database searches were performed using sequences catalogued at either IMG/M (http://img.jgi.doe.gov/m.; gene object ID) or GenBank (http://www.ncbi.nlm.nih.gov/; accession number).
[c]BLASTN uses an identity matrix for nucleotide comparisons, see http://www.ncbi.nlm.nih.gov/BLAST/; total length of query sequence was 163 bp for 16S rRNA, 121 bp for *acnB*, 162 bp for *ICL*, 157 bp for *sdhB*, 141 bp for cyb/b_6 (corresponding to length of amplicon generated in qRT-PCR).
[d]*E*-value or expect value describes the significance of the match between two sequences, see http://www.ncbi.nlm.nih.gov/BLAST/.

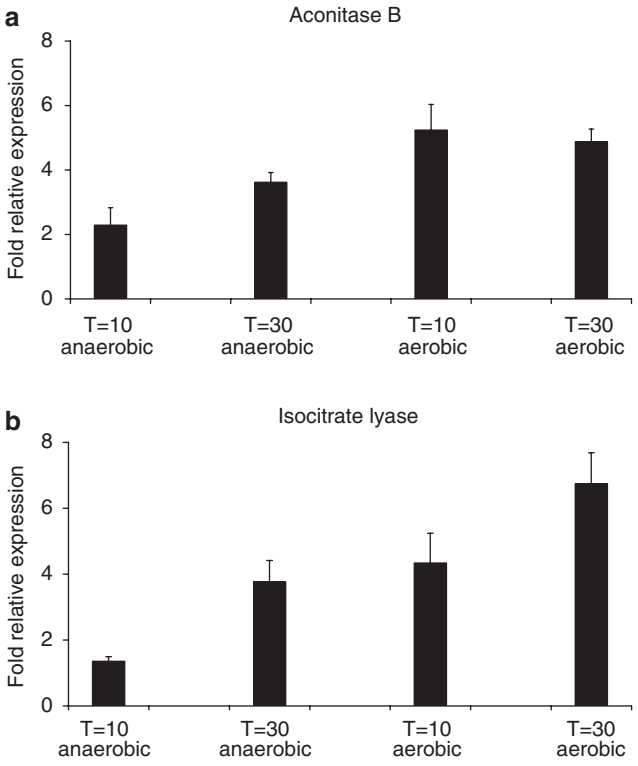

Figure 1 Expression of the genes encoding for (**a**) *acnB* and (**b**) *ICL* in the glyoxylate cycle of *Accumulibacter* subjected to sequential anaerobic:aerobic conditions. Transcript profiles were determined with acetate as the C source under anaerobic conditions and intracellular PHA as the C source under aerobic conditions and compared to transcripts expressed under baseline anaerobic conditions in the absence of a C source. Fold relative expression was determined with respect to the 16S rRNA gene.

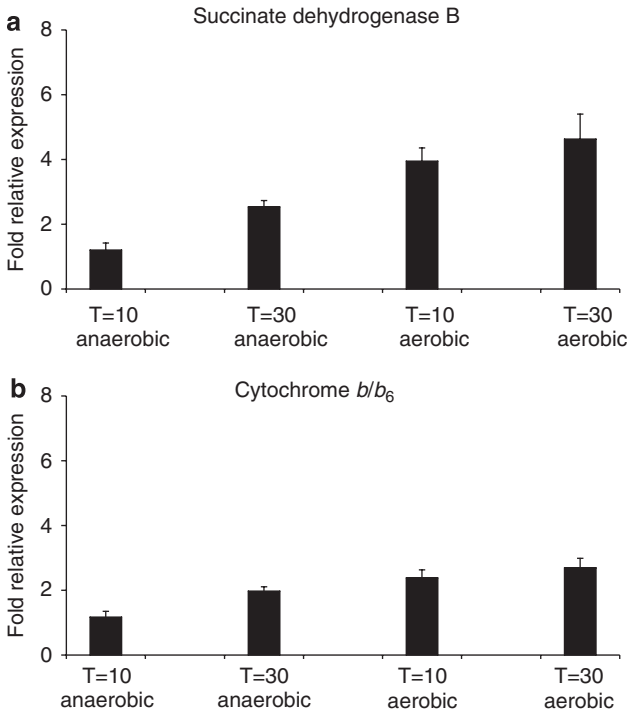

Figure 2 Expression of the genes encoding for (**a**) *sdhB* and (**b**) *cyb/b*6 in the electron transport chain of *Accumulibacter* subjected to sequential anaerobic:aerobic conditions. Transcript profiles were determined with acetate as the C source under anaerobic conditions and intracellular PHA as the C source under aerobic conditions and compared to transcripts expressed under baseline anaerobic conditions in the absence of a C source. Fold-relative expression was determined with respect to the 16S rRNA gene.

30 min; Figure 1a) under aerobic conditions compared to baseline expression. Similarly *ICL* was upregulated under anaerobic conditions by 1.4-and 3.8-fold after 10 and 30 min, respectively (Figure 1b). Under aerobic conditions the profile of *ICL* further increased by 4.3-fold after 10 min and 6.7-fold after 30 min (Figure 1b).

sdhB and *cyb/*b_6 transcripts increased under anaerobic conditions in the presence of acetate and were enhanced further in the presence of oxygen. An increase in *sdhB* (1.2-fold after 10 min and 2.5-fold after 30 min; Figure 2a) and *cyb/*b_6 (1.2-fold after 10 min and 2.0-fold after 30 min; Figure 2b) transcripts was observed under anaerobic conditions. The transcript profile data show that *sdhB* and *cyb/*b_6 transcripts were further enhanced during aeration, with *sdhB* increasing 3.9-fold after 10 min and 4.6-fold after 30 min (Figure 2a) and *cyb/*b_6 increasing 2.4-fold after 10 min and 2.7-fold after 30 min (Figure 2b).

Discussion

Accumulibacter was the dominant organism in the PAO sludge community (79(±5) to 85(±2)% of *Bacteria*) and demonstrated acetate uptake and assimilation as PHA (HB/HV) in the absence of external electron acceptors. Previous bioinformatic analyses suggest that acetate is activated via acetyl coenzyme A synthase under relatively low acetate conditions or by the action of acetate kinase and phosphotransacetylase in the presence of higher concentrations of acetate in *Accumulibacter*. The PHAs generated from acetate, polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) are likely synthesized by the same genes (*phaABC*) (Garcia Martin *et al.*, 2006; Figure 3).

Anaerobic consumption of glycogen (stored glucose) during acetate assimilation to PHA and the cessation of glycogen consumption in the presence of an inhibitor of GAPDH were observed. This suggests that glycolysis can generate reducing power for PHA generation in *Accumulibacter* (Figure 3). Glycolysis activity in *Accumulibacter* is consistent with previous reports that enriched *Accumulibacter* cultures catabolize glycogen under anaerobic conditions (Lu *et al.*, 2006) and as reported in models of anaerobic PAO metabolism (Filipe *et al.*, 2001). Glycolysis is likely to proceed via the EMP pathway, as genes for this pathway are all present, whereas key genes within the ED (Garcia Martin *et al.*, 2006)

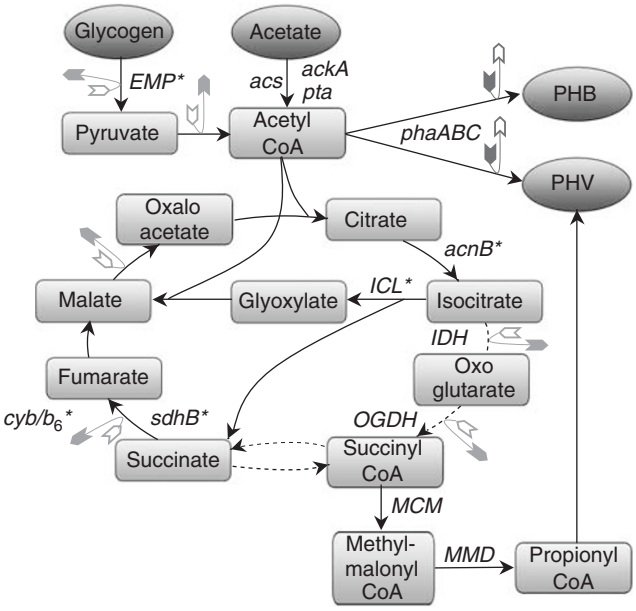

Figure 3 Hypothetical model of anaerobic central carbon metabolism in *Accumulibacter*. Genes or pathways marked with an asterisk (*) were investigated using inhibitors or gene expression analysis. Solid lines indicate probable carbon flux, whereas broken lines represent possible carbon flux not investigated in this study. Other genes are included based on genome sequencing of *Accumulibacter*. Filled arrows (▰) represent reduced electron carriers (NADH2, NADPH2 or FADH2) and unfilled arrows (▱) represent oxidized electron carriers (NAD+, NADP+ or FAD+). Green arrows (▱, ▰) depict reactions that reduce electron carriers/generate reducing power. Reactions that oxidize electron carriers/consume reducing power are depicted by red arrows (▰, ▱). PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate.

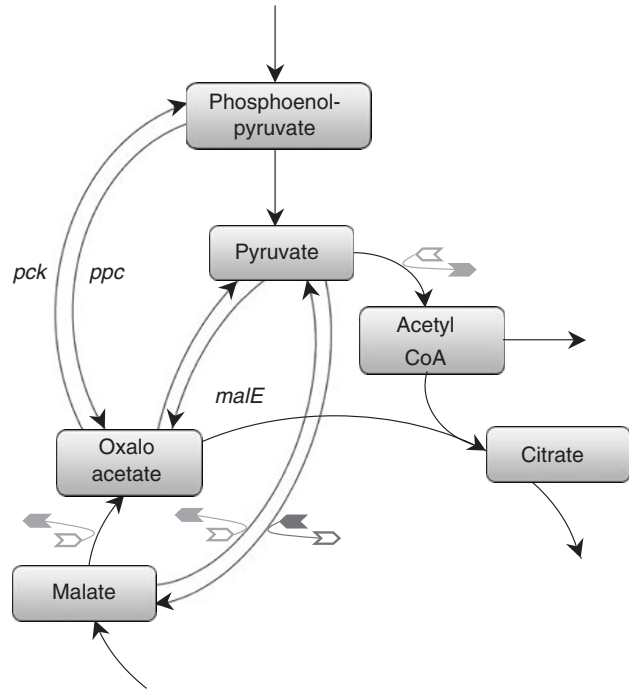

Figure 4 Hypothesized malate–pyruvate and phosphoenolpyruvate–oxaloacetate cycling potential in *Accumulibacter* based on gene sequence data. Filled arrows (▰) represent reduced electron carriers (NADH2, NADPH2 or FADH2) and unfilled arrows (▱) represent oxidized electron carriers (NAD+, NADP+ or FAD+). Green arrows (▱, ▰) depict reactions that reduce electron carriers/generate reducing power. Reactions that oxidize electron carriers/consume reducing power are depicted by red arrows (▰, ▱).

and PP pathway in sequenced strains of *Accumulibacter* are not.

Anaerobic carbon flux through the glyoxylate cycle in *Accumulibacter* (Figure 3) is consistent with the significant decrease in acetate uptake rates and PHA production in the presence of metabolic inhibitors of aconitase (fluoroacetate) and *ICL* (3-nitropropionate and itaconate). *ICL* and *MS* genes both unique to the glyoxylate cycle were subsequently identified demonstrating the potential for activity of this cycle. Expression of *acnB* and *ICL* were upregulated in *Accumulibacter* in the presence of acetate under anaerobic conditions providing further evidence of anaerobic glyoxylate cycle activity. Glyoxylate cycle activity is essential for microorganisms that utilize acetate as a sole carbon source (Kornberg, 1966). In *Cornyebacterium glutamicum* acetate–glucose mixtures are co-utilized and the glyoxylate cycle has been shown to be essential for its optimal growth on these substrates (Wendisch *et al.*, 2000). PHA production from acetate in PAOs requires reducing power in excess of that generated by glycogen catabolism (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). PHA acts as an electron sink in *Accumulibacter* and C flux of acetate and/or glucose through the glyoxylate cycle would yield the additional reducing power predicted to be necessary for the PHA production observed in PAOs.

Interconversion of C_3 carbon intermediates of the EMP pathway with C_4 carbon intermediates of the glyoxylate cycle may consume reducing power in the forward flux direction and generate reducing power in the backward flux direction (Sauer and Eikmanns, 2005). The *malE* gene encoding for an enzyme that mediates interconversion of malate and pyruvate (concomitantly carboxylates/decarboxylates oxaloacetate) in both the forward and reverse directions has been identified within the *Accumulibacter* genome (Garcia Martin *et al.*, 2006). The glyoxylate cycle intermediate malate may reenter a pyruvate pool destined for conversion to acetyl-CoA in a pyruvate–malate cycle (Petersen *et al.*, 2000; Lu *et al.*, 2002). Anaerobic activity of this cycle could provide a mechanism to balance the amount of reducing power generated by *Accumulibacter* (Figure 4). The genes *ppc* and *pck* encoding for the PEP-carboxylase and PEP-carboxykinase enzymes, respectively, were identified in this study in the genome of *Accumulibacter* and may provide an alternative link between central pathways in *Accumulibacter* (Figure 4). Application of labelled carbon techniques that enable determination of complex metabolic flux networks (Klapa *et al.*, 2003) in highly enriched cultures of *Accumulibacter* would be one strategy to determine bidirectional fluxes of carbon through these cycles.

Some metabolic models of anaerobic acetate metabolism in PAOs have overlooked the production of polyhydroxvalerate (PHV) due to the failure to assay for this type of PHA or due to detection of relatively minor amounts (Smolders *et al.*, 1994a; Filipe *et al.*, 2001; Zeng *et al.*, 2003). Zeng *et al.* (2003) suggested that proliferation of glycogen non-polyphosphate accumulating organisms (GAOs) known to produce relatively high amounts of PHV in EBPR sludge communities performing good P_i removal could explain the presence of PHV in enrichment cultures thought to be solely dominated by PAOs. However, in this study a substantial level of PHV (10%) was measured in high enrichment cultures of *Accumulibacter* ($\geqslant$79%), where GAO populations were low (4–6% of *bacteria*). In *Accumulibacter*, succinate may leave the glyoxylate cycle and be degraded to propionyl-CoA and condense with acetyl-CoA to form HV monomers, explaining anaerobic PHV production in these PAOs. Bioinformatic analysis of sequenced strains of *Accumulibacter* indicates that propionyl-CoA formation could proceed via methylmalonyl CoA mutase conversion of succinyl-CoA to methylmalonyl-CoA and decarboxylation of methylmalonyl-CoA by methylmalonyl decarboxylase to propionyl-CoA (Garcia Martin *et al.*, 2006; Figure 3).

Oxidation of succinate to fumarate in the absence of external electron acceptors in EBPR sludge communities has been difficult to explain due to a requirement for a terminal electron acceptor more electropositive than that of the fumarate/succinate electron carrier (Mino *et al.*, 1998). Despite this, evidence of the activity of succinate dehydrogenase in EBPR sludge communities performing good P_i removal has been demonstrated in the presence of acetate (Pereira *et al.*, 1996; Louie *et al.*, 2000) and propionate (Lemos *et al.*, 2003). Anaerobic succinate dehydrogenase activity was observed in *Accumulibacter* as determined by inhibition with malonate and the upregulation of *sdhB* as determined by qRT-PCR assays (Figure 3). No evidence was provided to support the reduction of fumarate to succinate in the *Accumulibacter* enrichment due to the insignificant effect of the fumarate reductase inhibitor observed on acetate uptake rates (Burow *et al.*, 2008) and PHA production.

Downhill transport of electrons from the fumarate/succinate couple cannot occur in the absence of external electron acceptors. However, uphill electron transport to electron carriers at the cost of PMF (Elbehti *et al.*, 2000) could explain carbon flux mediated by succinate dehydrogenase in *Accumulibacter* under anaerobic conditions. Garcia Martin *et al.* (2006) proposed that anaerobic activity of a novel *cyb/b*$_6$ fusion protein with hypothesized quinone oxidase activity could facilitate reverse electron transport driven by the PMF. The activity of quinone oxidase was inferred in *Accumulibacter* from the observed upregulation of *cyb/b*$_6$ under anaerobic conditions in the presence of acetate. Anaerobic acetate uptake in *Accumulibacter* has previously been shown to require a PMF (Saunders *et al.*, 2007; Burow *et al.*, 2008). A substantial reduction in PHA production occurred in the *Accumulibacter* enrichment when the PMF was uncoupled using CCCP. Although the observations made here are consistent with quinone oxidase activity in *Accumulibacter*, further research efforts should investigate the quinone oxidase protein to confirm that it is functioning anaerobically.

Activity of the full oxidative TCA cycle under anaerobic conditions in PAOs is a controversial hypothesis in EBPR research (Seviour *et al.*, 2003). Our efforts to clarify its activity under these conditions were unsuccessful (Figure 3). Attempts to target enzymes unique to the TCA cycle (isocitrate dehydrogenase, *IDH* or oxoglutarate dehydrogenase, *OGDH*) in qRT-PCR analysis failed; likely due to the intra-strain variation of gene sequences in *Accumulibacter* (Table 3). Several primer sets designed (based on near-complete genome sequences of two strains of *Accumulibacter*) to target regions of *IDH* ($n=6$) or *OGDH* ($n=6$) unique to *Accumulibacter* did not generate specific amplicons suitable for qRT-PCR analysis.

In PAOs, TCA pathways including the TCA cycle are thought to operate under aerobic conditions primarily for macromolecule synthesis (proteins, nucleic acids, lipids and polysaccharides) and cell growth, in contrast to their proposed primary role under anaerobic conditions to supply the cell with reducing power for PHA formation (Smolders *et al.*, 1994b; Pereira *et al.*, 1996). Stoichiometric data generated from PAO enrichment cultures propose that some PHA is channelled through the TCA cycle to generate macromolecules and energy via the electron transport chain, whereas another fraction is channelled through the glyoxylate cycle and gluconeogenesis to replenish glycogen (Smolders *et al.*, 1994b; Filipe and Daigger, 1998). Aerobic upregulation of *acnB* and *ICL* in *Accumulibacter* with intracellular PHA as the sole C source indicates glyoxylate cycle activity may be important for flux of C derived from PHA depolymerization. The *sdhB* and *cyb/b*$_6$ genes were also upregulated aerobically. As *Accumulibacter* is an aerobic heterotroph that possesses the full complement of genes in the TCA cycle (Seviour *et al.*, 2003; Garcia Martin *et al.*, 2006) it is likely that the TCA cycle operates aerobically together with the glyoxylate cycle. Aerobic operation of the glyoxylate cycle, which bypasses the decarboxylation steps of the TCA cycle, could be important for regulating the amount of stored carbon that is oxidized to CO_2 for energy and growth and the amount used for replenishment of glycogen.

Accumulibacter are aerobic heterotrophs that proliferate under unique anaerobic:aerobic, feast:famine cycling conditions because of their capability to transform intracellular PHA. Efficient storage and utilization of PHA are of key importance to their

dominance under these conditions. It is likely that flexibility in C metabolism via C flux through the glycolysis pathway, glyoxylate and TCA cycles and interconversion of intermediates between them is essential for *Accumulibacter* to respond to the changing electron acceptor and donor conditions in the EBPR process.

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