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Minireview

Microbiology of '*Candidatus* Accumulibacter' in activated sludge

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Summary

'*Candidatus* Accumulibacter' is a biotechnologically important bacterial group that can accumulate large amounts of intracellular polyphosphate, contributing to biological phosphorus removal in wastewater treatment. Since its first molecular identification more than a decade ago, this bacterial group has drawn significant research attention due to its high abundance in many biological phosphorus removal systems. In the past 6 years, our understanding of Accumulibacter microbiology and ecophysiology has advanced rapidly, largely owing to genomic information obtained through shotgun metagenomic sequencing efforts. In this review, we focus on the metabolism, physiology, fine-scale population structure and ecological distribution of Accumulibacter, aiming to integrate the information learned so far and to present a more complete picture of the microbiology of this important bacterial group.

Introduction

Phosphorus (P), a growth-limiting nutrient in many surface water bodies, is an important aspect in wastewater management, as extensive evidence indicated that P is a leading factor causing surface water eutrophication. Through years of practice, wastewater engineers have learned to utilize an interesting physiology exhibited by some microorganisms in activated sludge processes to reduce soluble P concentrations in wastewater. Under cyclic anaerobic and aerobic conditions, these organisms 'luxuriously' take up more inorganic phosphate (P_i) than their metabolic demand and store it as intracellular polyphosphate (polyP). Therefore, this process was named 'enhanced biological phosphorus removal (EBPR)' and these organisms were called 'polyphosphate-accumulating organisms (PAOs)' (Mino *et al.*, 1998). A traditional EBPR configuration consists of an anaerobic (lack of any external electron acceptor) zone followed by an aerobic zone, and P is ultimately removed from the system by wasting sludge with high polyP contents (Fig. 1A). Metabolic models have been proposed to describe this process based on chemical profiles in the bulk liquid and within the biomass (Fig. 1B) [see also the review by Oehmen and colleagues (2007)]. Anaerobically, intracellular polyP is degraded to generate ATP, the utilization of which provides energy for organic substrate uptake, such as volatile fatty acids (VFAs), and leads to P_i release into the bulk liquid. VFAs are then converted to polyhydroxyalkanoate (PHA), a polymer more reduced than VFAs. The reducing power for this conversion is thought to be provided by the degradation of intracellular glycogen and/or the tricarboxylic acid (TCA) cycle. Under subsequent aerobic conditions, PHA is oxidized and P_i is taken up to form polyP, with concomitant glycogen replenishment and cell growth. Therefore, EBPR sludge often exhibits characteristic cyclic transformations of polyP, PHA and glycogen (Fig. 1B). Despite several decades of EBPR application in the wastewater treatment industry, this economical and environmentally friendly biotechnology still suffers from unpredictable and sometimes unexplained failures (Neethling *et al.*, 2005). Understanding 'by whom, why and how polyP is accumulated' should provide theoretical ground for more rational design, effective operation and troubleshooting of this important engineered ecosystem.

To date, no confirmed PAO exhibiting all of the characteristics observed during EBPR (Fig. 1B) has been cultivated in isolation. Instead, investigation using culture-independent approaches led to the identification of bacteria phylogenetically affiliated with the *Rhodocyclus* group of the *Betaproteobacteria* (Bond *et al.*, 1995; Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000) as numerically dominant PAOs in lab-scale EBPR reactors, and members of this group have been named '*Candidatus* Accumulibacter phosphatis' [henceforth referred to as the

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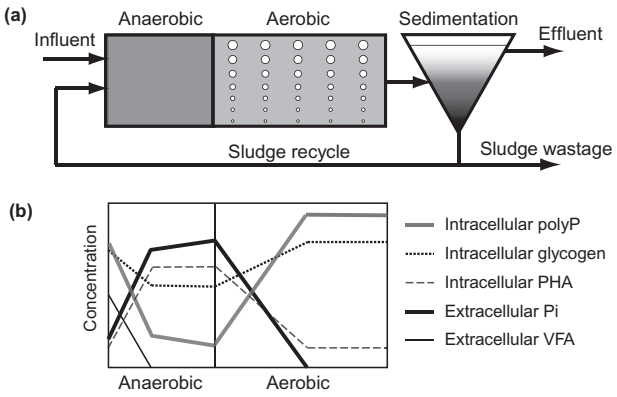

Fig. 1. Schematic representation of a traditional EBPR process with an anaerobic/aerobic configuration (A) and characteristic chemical profiles during an EBPR cycle exhibited by sludges fed with VFAs (B). The figure was modified from an earlier review by Blackall and colleagues (2002). Note that the cycling of glycogen was minimal in continuously aerated sludges with feast and famine phases (Ahn *et al*., 2007), and the *Tetrasphaera*-related PAOs within the Actinobacteria are specialized in using amino acids and do not appear to accumulate PHAs (Kong *et al*., 2005; Nielsen *et al*., 2010).

Accumulibacter (lineage)] (Hesselmann *et al*., 1999). Three fluorescent *in situ* hybridization (FISH) probes were designed and applied simultaneously to target the 16S rRNA of this bacterial group, and were later referred to as the PAOmix probes (Crocetti *et al*., 2000). Since then, Accumulibacter has been repetitively detected in reactors fed with acetate or propionate, operated in laboratories across different continents (Oehmen *et al*., 2007). Colocalizing FISH-positive cells with chemical stains demonstrated the *in situ* physiology of polyP and PHA accumulation by Accumulibacter from lab-scale activated sludge (Liu *et al*., 2001). A survey of full-scale EBPR treatment plants confirmed the abundance of Accumulibacter and its involvement in P removal (Zilles *et al*., 2002a).

In the past six years, tremendous progress has been made, especially with the sequencing of an Accumulibacter genome, followed by post-genomics research, which greatly advanced our understanding of how this biotechnologically important and ecophysiologically interesting bacterial group performs during wastewater treatment. Although other bacteria, such as Actinobacteria, were also identified as important PAOs in some EBPR systems [see a good review on Actinobacteria in activated sludge (Seviour *et al*., 2008)], our review only focuses on Accumulibacter, since most research efforts to date have focused on this lineage.

Genomic blueprint

One of the most exciting and fruitful recent studies on EBPR included the shotgun metagenomic sequencing of two lab-scale EBPR sludges: an acetate-fed sludge enriched with ~80% Accumulibacter from Wisconsin, USA (US) and a propionate-fed sludge enriched with ~60% Accumulibacter from Queensland, Australia (OZ) (Garcia Martin *et al*., 2006). Despite the operational and geographical differences, the dominant Accumulibacter strains in these two reactors shared higher than 95% nucleotide sequence identity over 79% of the reconstructed US genome. Low-abundance Accumulibacter strains were also detected, with partial coverage of their genomes, which were estimated to be ~15% divergent at the nucleotide level from the dominant strains. With later additional sequencing, assembly and annotation efforts, the complete genome of the dominant US Accumulibacter strain was obtained (clade IIA str. UW-1, NCBI taxon ID 522306) and publicly available at IMG (http://img.jgi.doe.gov). The genome is comprised of a 5.06 Mbp chromosome and three plasmids. Altogether, the 5.31 Mbp genome contains 4735 protein-coding genes. More than three thousand genes have function prediction, allowing a nearly complete metabolic reconstruction (Fig. 2) that helped to resolve some long-existing debates regarding EBPR metabolism. The genomic blueprint also reflects some key features of Accumulibacter that enable it to thrive under EBPR conditions and reveals its lifestyle outside wastewater treatment environments. These are discussed in more detail throughout this review.

EBPR metabolism and regulation

After this landmark metagenomic analysis, post-genomics research tools have been applied to Accumulibacter-dominated EBPR sludges to study gene expression and regulation. For example, Burow and colleagues (2008a) and He and McMahon (2011) used quantitative reverse transcription PCR (qRT-PCR) to monitor transcription of key genes associated with EBPR metabolism. On a larger scale, microbial community gene expression was studied at the transcription level (metatranscriptomics) using microarrays (He *et al*., 2010a), and at the translation level (metaproteomics) using 2D-PAGE combined with tandem mass spectrometry (MS) (Wilmes *et al*., 2008a; Wexler *et al*., 2009) and liquid chromatography combined with tandem MS (Wilmes *et al*., 2008b). In this section, we integrate the genomics and post-genomics research with traditional kinetic and stoichiometric studies conducted on Accumulibacter-enriched sludges to discuss its metabolism and regulation relevant to EBPR (Fig. 2).

Carbon metabolism

Acetate is a model carbon source used in EBPR research. In *Escherichia coli* acetate is transported through a symporter acetate permease (ActP) (Gimenez *et al*., 2003). A

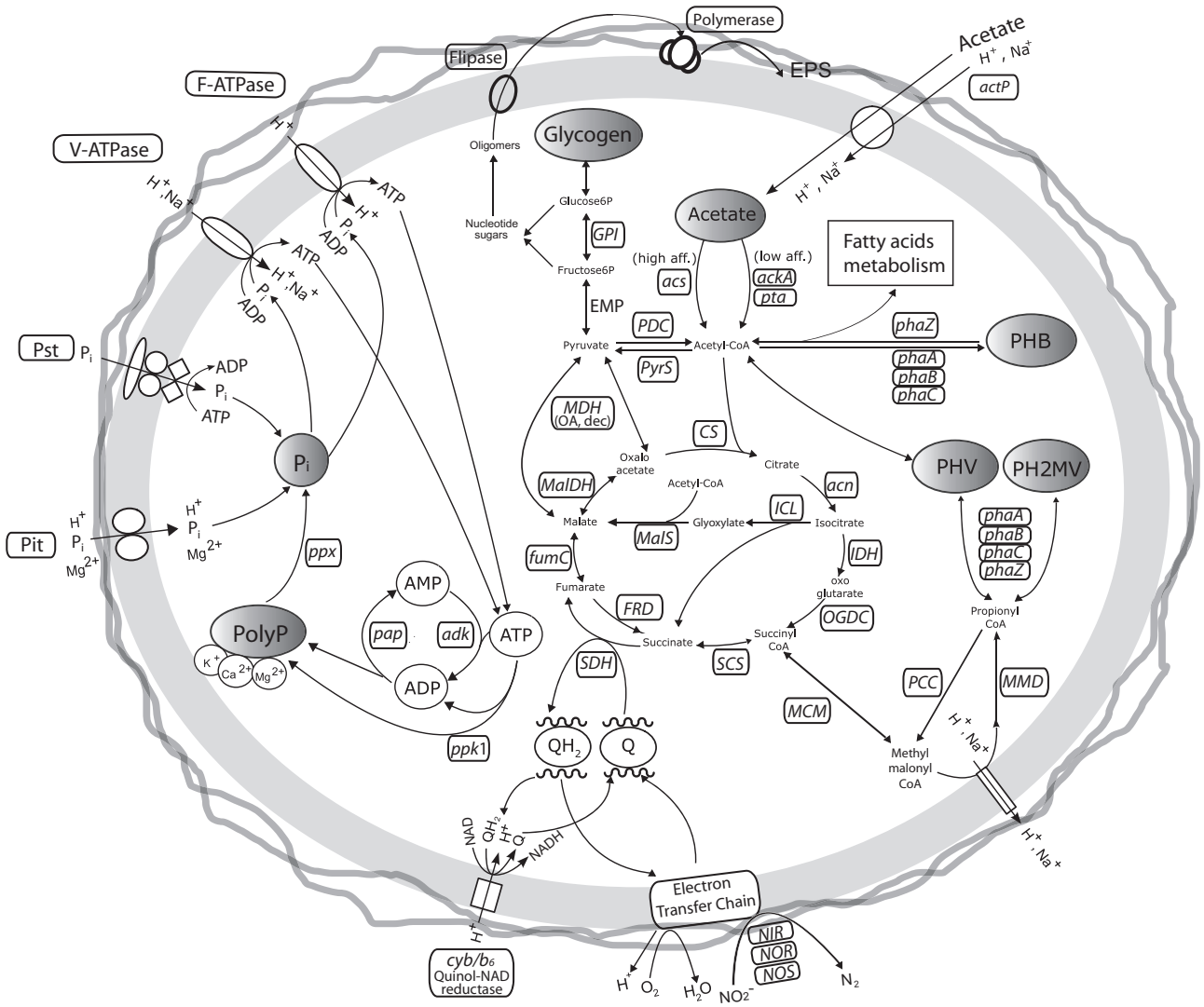

Fig. 2. EBPR metabolic map inferred from the Accumulibacter clade IIA genome, adapted from Garcia Martin and colleagues (2006). Abbreviations: *acs*, acetyl-CoA synthase; *ackA*, acetate kinase A; *pta*, phosphotransacetylase; *GPI*, glucose-6-phosphate isomerase; EMP, Embden–Meyerhof–Parnas pathway; *PyrS*, pyruvate synthase; *PDC*, pyruvate dehydrogenase complex; *phaA*, acetyl-CoA acetyl transferase; *phaB*, acetoacetyl-CoA reductase; *phaC*, PHA synthase; *phaZ*, PHA depolymerase; *CS*, citrate synthase; *acn*, aconitase; *IDH*, isocitrate dehydrogenase; *OGDC*, 2-oxoglutarate dehydrogenase; *SCS*, succinyl-CoA synthetase; *SDH*, succinate dehydrogenase; *fumC*, fumarase C; *MalDH*, malate dehydrogenase; *ICL*, isocitrate lyase; *MalS*, malate synthase; *MDH* (OA dec), malate dehydrogenase (oxaloacetate-decarboxylating); *FRD*, fumarate reductase; *MCM*, methylmalonyl-CoA mutase; *PPC*, propionyl-CoA carboxylase; *MMD*, methylmalonyl-CoA decarboxylase; F-ATPase, F_0F_1-type ATP synthase; V-ATPase, Archaeal/vacuolar-type H^+-ATPase; *ppk1*, polyphosphate kinase 1; *adk*, adenylate kinase; *pap*, polyP-AMP phosphotransferase; *ppx*, exopolyphosphatase; *NIR*, nitrite reductase; *NOR*, nitric oxide reductase; *NOS*, nitrous oxide reductase.

homologue of *actP* identified in the Accumulibacter genome was predicted to transport acetate across the cell membrane using the proton and/or sodium ion (Na^+) gradient (Fig. 2); and its expression was detected by metaproteomics (Wilmes *et al*., 2008b). Similar to ActP in *E. coli*, acetate uptake in an Accumulibacter-dominated sludge was significantly inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton motive force uncoupler, but not by monensin, a Na^+ gradient disrupter, suggesting that acetate transport by ActP was energized by the proton motive force, instead of a Na^+ gradient (Saunders *et al*., 2007; Burow *et al*., 2008b). However, because the inhibition of a particular pathway may promote other pathways not regularly employed by the microorganisms (Oehmen *et al*., 2010c), and enzyme inhibitors sometimes are not specific, results generated using inhibitors need to be interpreted cautiously.

Like in *E. coli*, Accumulibacter *actP* clusters together with *acs*, a gene encoding the high-affinity acetyl-CoA synthase (ACS) for acetate activation to acetyl-CoA. In addition, Accumulibacter also possesses the low-affinity acetate kinase/phosphotransacetylase (AckA/Pta)

pathway for acetate activation. Because the half-saturation concentration of acetate is generally > 1 mM when acetate is activated through the AckA/Pta pathway, Hesselmann and colleagues (2000) suggested that acetate activation was mainly through ACS. Consistent with this, the metaproteomics revealed that although both high- and low-affinity activation pathways were expressed, the former was preferentially used, as indicated by its much higher expression level than the latter (Wilmes *et al*., 2008b).

Anaerobically, acetyl-CoA is converted to PHAs, using the reducing equivalents in the form of NAD(P)H provided by glycogen degradation [the Mino model (Mino *et al*., 1987)] and/or the TCA cycle [the Comeau/Wentzel model (Comeau *et al*., 1986; Wentzel *et al*., 1986)]. Glycogen degradation through the Embden–Meyerhof–Parnas (EMP) (Mino *et al*., 1987) or the Entner Doudoroff (ED) (Maurer *et al*., 1997; Hesselmann *et al*., 2000) pathway has been long debated. Which pathway is employed is important for the cellular energy budget, as the EMP pathway generates more ATP than the ED pathway. The Accumulibacter genome sequence unambiguously indicated the employment of the EMP pathway, because the key gene in the ED pathway was absent. This is supported by the detection of gene expression in the EMP pathway at both transcription (He *et al*., 2010a) and translation levels (Wilmes *et al*., 2008a,b; Wexler *et al*., 2009).

Another controversy surrounds the contribution of the TCA cycle and its mode of operation in EBPR anaerobic metabolism. Three modes of anaerobic TCA cycle operation have been proposed for EBPR, namely the full (Comeau *et al*., 1986), partial (bypass through the glyoxylate shunt) (Louie *et al*., 2000) and split modes (right, or the oxidative arm of the TCA cycle operating forward and left, or the reductive arm operating backward through the succinate–propionate pathway) (Hesselmann *et al*., 2000) [see reviews by Oehmen and colleagues (2007) and Zhou and colleagues (2010)]. Genes involved in these three pathways were identified in the Accumulibacter genome (Fig. 2) (Garcia Martin *et al*., 2006), and it is just a question of if and how Accumulibacter use them anaerobically. A key enzyme in the TCA cycle pathway, succinate dehydrogenase (SDH), was anaerobically expressed, and when SDH was inhibited, acetate uptake and PHA production rates decreased, suggesting the involvement of the TCA cycle in anaerobic NAD(P)H generation (Burow *et al*., 2008a). By modelling the carbon fluxes in glycogen-depleted sludges, Zhou and colleagues (2009) demonstrated that the contribution of anaerobic TCA cycle depended on glycogen content and suggested that the full TCA cycle was operative anaerobically. However, $FADH_2$ generated by SDH cannot be re-oxidized in the absence of external electron acceptors, causing $FADH_2$ accumulation and disabling the full TCA cycle. A solution to this may be the novel cytochrome b/b_6 (cyb/b_6) identified in the Accumulibacter genome which was predicted as a quinol-NAD(P) reductase capable of transferring electrons from $FADH_2$ to NAD(P) at the expense of a proton gradient (Garcia Martin *et al*., 2006). Further investigation is needed to verify the postulated function of this novel cyb/b_6. On the other hand, the partial TCA cycle can bypass SDH and the accumulation of $FADH_2$ through the glyoxylate shunt pathway, for which the expression of a key gene encoding isocitrate lyase (ICL) was detected at both transcription (Burow *et al*., 2008a; He and McMahon, 2011) and translation levels (Wilmes *et al*., 2008b; Wexler *et al*., 2009). Burow and colleagues (2008a) observed an increase in *ICL* transcripts during the anaerobic phase, and decreases in acetate uptake and PHA synthesis rates when the glyoxylate shunt pathway was inhibited, and therefore suggested a role of this pathway in anaerobic PHA formation. However, Wexler and colleagues (2009) found that ICL levels were higher in the aerobic phase, and proposed that the glyoxylate shunt pathway was unlikely important in the anaerobic phase. Finally, in the split TCA cycle, $FADH_2$ can be re-oxidized by fumarate reductase (FRD), and together with the succinate–propionate pathway via methylmalonyl-CoA, this pathway results in PHV formation (Fig. 2), in addition to PHB, the dominant form of PHA when fed with acetate. The expression of FRD and a few other genes in this pathway was detected at levels of transcription (He and McMahon, 2011; He *et al*., 2010a) and translation (Wilmes *et al*., 2008b). However, Burow and colleagues (2008b) suggested a lack of FRD activity due to the very low PHV : PHB ratio measured in their sludge. Taken together, these studies indicated the involvement of the anaerobic TCA cycle, likely through multiple pathways. The existing discrepancies in the major mode of operation highlight the metabolic versatility and flexibility of Accumulibacter under different local conditions, and may partly arise from different Accumulibacter strains dominating in these sludges (discussed later).

The expression of other key metabolic pathways in EBPR carbon metabolisms, such as the formation and degradation of PHA and glycogen, was detected by community transcriptomic and proteomic analyses. Notably, Wilmes and colleagues (2008b) identified a large number (702) of proteins from Accumulibacter, well representing pathways described in EBPR metabolic models. In addition, they found high levels of proteins involved in fatty acid metabolism and therefore hypothesized fatty acid β oxidation as a novel mechanism for NAD(P)H generation for PHA synthesis. However, further experiments by Wexler and colleagues (2009) showed an increased fatty acid content in the anaerobic phase, contrasting to the hypothesized role of fatty acids in anaerobic NAD(P)H

generation. Wexler and colleagues therefore speculated that anaerobic increases of fatty acids were associated with synthesis of phospholipids for the cell membrane or to encapsulate intracellular PHA granules. Nevertheless, the high expression levels of fatty acid metabolic genes warrant future investigation to elucidate their functions in EBPR metabolism.

Polyphosphate metabolism

Two phosphate permeases in the low-affinity inorganic P_i (Pit) system and three highly conserved clusters of the high-affinity P_i-specific (Pst) system were found in the Accumulibacter genome (Garcia Martin *et al*., 2006). This redundancy might be Accumulibacter's adaptation to the EBPR environment to transport P_i effectively, irrespective of external P_i concentration. Based on P_i concentrations that normally occur during an EBPR cycle, Garcia Martin and colleagues (2006) postulated that the Pit system was used under high P_i concentrations, while the Pst system was suppressed during EBPR operation until P_i levels became very low. However, Burow and colleagues (2008b) proposed that the ATP-driven Pst system was used for P_i uptake even under relatively high P_i concentrations, as P_i uptake was inhibited by *N*,*N*′-dicyclohexylcarbodiimide (DCCD), which can covalently modify the ATPase, and thus inhibit the generation of ATP that energizes the Pst system. Since P_i needs to be converted to ATP before being incorporated into polyP (Fig. 2), the inhibited ATPase activity would also further interfere with the formation of polyP from P_i, therefore slowing down P_i uptake. In addition, as a covalent carboxyl-modifying agent, DCCD also inhibits a number of membrane-bound enzymes in the electron transfer chain (Yagi, 1987), capable of interrupting the aerobic processes and P_i uptake regardless of which P_i transport system is used. Therefore, the inhibited P_i uptake by DCCD under high P_i concentrations does not necessarily indicate the utilization of Pst under that condition. Nevertheless, the Pst component genes were transcribed (He *et al*., 2010a) and translated in both anaerobic and aerobic phases (Wilmes *et al*., 2008b), and its unexpectedly high protein level in the anaerobic phase led Wilmes and colleagues (2008b) to postulate a role of Pst in P_i efflux coupling with ATP generation. However, alternative explanations could be a protein carryover from the aerobic phase or the regulation of Pst at the level of enzyme activity. Interestingly, one Pit permease was more abundant in the anaerobic phase, while the other Pit permease was only detected in the aerobic phase, possibly indicating different functions of these two permeases (Wilmes *et al*., 2008b): anaerobic P_i efflux with the proton motive force generation for acetate uptake through a proton-driven symporter as proposed earlier (Saunders *et al*., 2007; Burow *et al*., 2008b); and aerobic P_i uptake under relatively high P_i concentrations, respectively.

PolyP formation in model organisms, such as *E. coli*, is thought to occur primarily through a reversible reaction catalysed by polyphosphate kinase 1 (PPK1) using ATP as a substrate (Ahn and Kornberg, 1990). Intriguingly, the gene encoding guanosine 3′,5′-bis-diphosphate (ppGpp) synthase (RelA) is located next to *ppk1* and the exopolyphosphatase gene (*ppx*), forming a *ppx–ppk1–relA* cluster in the Accumulibacter genome (Garcia Martin *et al*., 2006). Under amino acid starvation, ppGpp accumulates and regulates global change in gene expression, known as the stringent response (Lamond and Travers, 1985). Elevated levels of ppGpp also inhibit PPX activity, favouring polyP formation (Kornberg *et al*., 1999), thus linking polyP metabolism and amino acid starvation. In addition to PPK1/PPX, an alternative route for polyP formation and degradation is also present in Accumulibacter (Garcia Martin *et al*., 2006). A polyP:AMP phosphotransferase (PAP) can phosphorylate AMP with polyP to generate ADP, which can then be converted to ATP and AMP by adenylate kinase (ADK), with resulting AMP reincorporated in the cycle to drive more polyP degradation (Fig. 2). The cycle can be reversed for polyP formation. Notably, both PPK1/RelA and PAP/ADK were detected by metaproteomic analysis (Wilmes *et al*., 2008b). Which route Accumulibacter primarily uses for polyP metabolism is still unclear.

Regulation of metabolism

The cyclic anaerobic and aerobic regimes coupled with alternating feast and famine conditions create a unique niche for organisms capable of taking up and storing organic carbon anaerobically using energy generated from subsequent oxidization of previously stored carbon. This competition strategy and sophisticated control of biochemistry has inspired research efforts to elucidate molecular-level regulation by comparing gene expression between phases during an EBPR cycle. He and colleagues (2010a) found that the majority of upregulated genes in the aerobic sample were predicted to encode functions such as transcription, translation, protein translocation and energy metabolisms, reflecting rapid growth of Accumulibacter shortly (15 min) after being switched to aerobic conditions. At the level of translation, Wilmes and colleagues (2008a,b) observed strong similarities between the aerobic and anaerobic protein profiles, with only 3–9% of total proteins exhibiting differential expression. The unanticipated small difference might be partly due to the rapid and cyclic environmental changes in EBPR, where it is probably more efficient for PAOs to minimize the flux of proteins regularly employed.

Changes in protein abundance would likely be more observable when operating conditions are disturbed. Another explanation might be the protein carryover from the previous set of conditions. To cope with this problem, Wexler and colleagues (2009) conducted proteomics on two lab-scale sludges incubated with radioactive ^{35}S-labelled methionine to restrict the analysis to newly synthesized proteins. They found more, although still not a large number of proteins exhibiting differential expression. In addition, while both sludges exhibited enhanced aerobic synthesis of enzymes in the glyoxylate shunt pathway, enzymes in the TCA cycle were upregulated in the anaerobic and aerobic phases for these two sludges, respectively, strongly suggesting variation in molecular-level regulation within the Accumulibacter lineage (discussed later).

Most mRNAs in bacteria have short half-lives [e.g. ~80% of mRNAs in *E. coli* or *Bacillus subtilis* exhibited half-lives on the order of a few minutes (Bernstein *et al*., 2002; Hambraeus *et al*., 2003)], and this was thought to be an adaptation to respond quickly to changing environments (Rauhut and Klug, 1999), such as during EBPR processes. In contrast, proteins are relatively stable [e.g. proteins in steady-state *E. coli* have half-lives on the order of hours (St. John *et al*., 1979)], and may carry over between phases, which last for only a few hours in EBPR. Therefore, the detection of transcripts is probably more sensitive than that of proteins to reflect the microbial response to rapid EBPR environmental changes, although the latter is hypothesized to be more accurate as a step closer to the final products.

Unlike the usual comparative (meta)transcriptomics or (meta)proteomics that contrast the expression profiles under two sets of conditions in parallel, the comparison between anaerobic and aerobic phases involves a temporal profiling. Depending on the patterns of expression dynamics, sampling at different time points as representatives of each phase may lead to different conclusions. Therefore, He and McMahon (2011) used qRT-PCR to monitor temporal expression profiles of key genes at a sub-hour resolution. They found that gene expression during an EBPR cycle was quite dynamic. Acetate addition in the anaerobic phase induced genes associated with the methylmalonyl-CoA pathway enabling the split mode of the TCA cycle. Components of the full TCA cycle were induced after the switch to aerobic conditions. *ICL* in the glyoxylate shunt pathway was induced by acetate addition and switching to aeration, with a higher induction by aeration. *ppk1* was expressed in both phases, but did not appear to be regulated by phosphate limitation. Rather *ppk1* was repressed by feeding acetate under aerobic conditions (discussed later). These findings suggest that several genes involved in EBPR are tightly regulated at the transcriptional level.

Although these post-genomics studies are informative, the associated limitations should not be overlooked. First, these studies measured the relative mRNA (or protein) concentration, which is a combined result of mRNA/protein responsive synthesis and intrinsic degradation. Second, they largely interpreted an increase in mRNA/protein abundance equates to upregulation, and therefore enzyme activity and operation of an associated pathway. However, alternate mechanisms may account for changes in mRNA/protein abundance. Genes could be regulated in response to environmental cues, making enzymes or pathways ready for a subsequent phase. For example, genes involved in phototrophy in marine bacteria were among the most highly expressed genes even at 3 h before sunrise (Frias-Lopez *et al*., 2008). This type of regulation is also likely under the rapid, cyclic and somewhat 'predictable' changes in EBPR, since no lag in chemical transformations has been observed upon the switch of phases. Lastly, biochemical pathways are also subjected to other regulations after transcription and translation steps, such as the regulation of enzyme activity. Therefore, caution should be taken when interpreting results from these studies.

Physiology

Electron donor and acceptor utilization

Determining the substrate spectrum available to Accumulibacter is essential to understanding ecological niches of this PAO in wastewater treatment environments where diverse carbon sources are present. Substrate specificity has been proposed as one of the most important factors determining EBPR community structure (Nielsen *et al*., 2010). By combining fluorescent *in situ* hybridization with microautoradiography (FISH-MAR), Kong and colleagues (2004) tested a range of ^{14}C-labelled organic substrates on samples collected from full-scale domestic wastewater treatment plants. They found that Accumulibacter was able to assimilate acetate, propionate, pyruvate and glutamate, but not formate, ethanol, butyrate, glucose, thymidine, oleic acid and several amino acids including leucine, glycine and aspartate. In contrast, Actinobacteria-related PAOs preferably use amino acids over acetate or propionate, and therefore do not accumulate PHAs (Kong *et al*., 2005). This partially explains the repetitive enrichment of Accumulibacter in lab-scale reactors where acetate and/or propionate are used as model substrates since they are the most commonly found VFAs in wastewater treatment systems.

Investigating Accumulibacter's ability to use nitrate or nitrite as alternative electron acceptors is important, particularly for treatment systems where simultaneous removal of P and nitrogen (N) is preferred. The FISH-MAR investigation with ^{33}P on full-scale sludges showed that

some, but not all, Accumulibacter cells were able to reduce nitrate and nitrite coupling with P uptake, suggesting that they were denitrifying PAOs (DPAOs) (Kong *et al*., 2004). However, conflicting evidence exists regarding the lineage's capabilities of nitrate reduction (discussed later).

Notably, nitrite has been shown to inhibit energy generation in many bacteria in general. Particularly for Accumulibacter-enriched sludges, several recent studies indicated that nitrite in the form of free nitrous acid (HNO_2) inhibited anoxic P uptake and denitrification (Zhou *et al*., 2007), as well as aerobic P uptake (Pijuan *et al*., 2010). The inhibitions occurred even at very low concentrations (on the order of 10^{-3} mg HNO_2-N l^{-1}), which adds challenges to design and operate simultaneous nitrification, denitrification and P removal, especially via the nitrite shortcut where the end-product of nitrification is nitrite.

Starvation

In wastewater treatment plants, the influent volume and organic substrate level fluctuates frequently. The diluted organic substrate concentrations after heavy rainfalls or the low organic loadings during weekends expose microorganisms to starvation, and have been associated with poor P removal performance (Carucci *et al*., 1999). Under starved conditions, cells go through endogenous processes, such as utilizing storage products (maintenance) and cell lysis resulting in a decrease of active cell mass (decay). Therefore starvation may affect the abundance and activity of key microbial players and the system performance. During an 8-day starvation of Accumulibacter-enriched sludge, polyP and glycogen were both utilized under anaerobic and anoxic conditions for maintenance, with negligible cell decay. Under aerobic starvation, glycogen but not polyP was used as the primary energy source for maintenance, with a higher cell decay rate. Intermittent aerobic–anaerobic starvation showed slower rates of glycogen and polyP degradation and negligible cell decay, therefore such alternating redox conditions could be used for long-term sludge storage (Lu *et al*., 2007). In another study with a longer starvation (up to 3–4 weeks), similarly, anaerobic starvation resulted in rapid utilization of polyP and glycogen with no significant cell decay. However, under aerobic starvation a higher decay rate with rapid utilization of PHA but slower utilization of glycogen and polyP was observed (Lopez *et al*., 2006). However, the identity of the dominant PAOs in this study was not reported.

Aerobic P release and continuously aerated EBPR

Physical separation of electron donor and acceptor is generally required for good P removal. As such, a typical phenotype of PAOs is anaerobic P release and aerobic P uptake. However, several studies showed that EBPR activities of Accumulibacter-dominated sludges were still maintained for a short period when acetate was fed under continuous aerobic conditions (Guisasola *et al*., 2004; Pijuan *et al*., 2005; 2006). Significant aerobic P release was observed, coupled with acetate uptake (feast phase), followed by P uptake after acetate was depleted (famine phase). However, aerobic EBPR could not be sustained for more than a few days under such conditions. Interestingly, the abundance of Accumulibacter did not change significantly after P removal failed, suggesting that a metabolic shift rather than a loss of Accumulibacter biomass accounted for deterioration in EBPR activity (Pijuan *et al*., 2006). To maintain EBPR metabolism, Ahn and colleagues (2007) tried an alternative feeding strategy by temporally separating acetate and P feeds, and were able to maintain good and stable EBPR under continuous aeration for several months with Accumulibacter being the dominant PAOs.

One consistent observation from these studies is the fact that the dissolved oxygen (DO) quickly dropped upon acetate addition due to microbial oxygen uptake. The relatively low DO levels in bulk solution might have caused oxygen transfer to the centre of sludge flocs to become diffusion-limited, likely creating local anaerobic conditions. This probably explains the discrepancy observed in a recent study (He and McMahon, 2011), where P release was almost completely inhibited under aerobic acetate addition when the DO was maintained at > 7 mg l^{-1}. Further qRT-PCR analysis showed that *ppk1* and methylmalonyl-CoA pathway transcription decreased while the aerobic TCA cycle exhibited higher expression, compared with the control sludge with anaerobic acetate addition, supporting the metabolic shift hypothesis suggested by Pijuan and colleagues (2006).

To avoid the oxygen limitation, Vargas and colleagues (2009) used an on/off controller to maintain the DO at 3.5–4.5 mg l^{-1} in a propionate-fed reactor. The Accumulibacter-dominated aerobic EPBR was operated for 46 days and was able to rapidly switch to the anaerobic/aerobic mode. The authors attributed the sustained EBPR activity to propionate being the carbon source, which results in PHV and PH2MV as dominant PHAs (Oehmen *et al*., 2005b), as compared with PHB produced from acetate used in previous continuous aeration studies. It is noteworthy that during long-term aerobic operation (Ahn *et al*., 2007; Vargas *et al*., 2009), significant PHA transformation between feast and famine phases was still observed, but glycogen transformation became negligible, suggesting that a portion of carbon substrate may have been oxidized through the TCA cycle, replacing the role of glycogen degradation in the generation of NAD(P)H needed for PHA synthesis.

Physiological differences and competition between Accumulibacter and glycogen-accumulating organisms (GAOs)

A notorious bacterial group often thriving in EBPR systems is the so-called glycogen-accumulating organisms (GAOs) that compete with PAOs for organic carbon substrates, through similar PHA and glycogen transformation but without polyP accumulation. Two major groups of GAOs were identified: '*Candidatus* Competibacter phosphatis' (Competibacter) (Crocetti *et al*., 2002) and *Alphaproteobacteria* GAOs, including *Defluviicoccus*-related GAOs (Wong *et al*., 2004; Meyer *et al*., 2006). These GAOs differ from Accumulibacter in detailed pathways used in carbon metabolism despite their similarities in end-products. For example, ^{13}C-labelled acetate nuclear magnetic resonance analysis suggested that Competibacter and *Defluviicoccus*-related GAOs used the ED pathway for glycogen degradation (Lemos *et al*., 2007), contrasting to the EMP pathway employed by Accumulibacter.

Further, unlike Accumulibacter, by which the proton gradient for acetate transport is thought to be primarily maintained by proton efflux in symport with P_i by Pit transporters (Saunders *et al*., 2007; Burow *et al*., 2008b), *Defluviicoccus*-related GAOs seem to maintain the proton gradient by FRD activity via the reductive arm of the TCA cycle, and also appear to generate a Na^+ gradient through decarboxylation of methylmalonyl-CoA (Burow *et al*., 2008b). In addition to these, Competibacter was also suggested to use proton efflux through F_1F_0-ATPase (Saunders *et al*., 2007). The bioenergetic difference in acetate transport may give Accumulibacter a competitive advantage at higher external pHs that demand more energy for acetate uptake. For example, increasing pH from 7 to 8 increased the abundance of Accumulibacter over *Alphaproteobacteria* GAOs fed on propionate and over Competibacter fed on acetate (Oehmen *et al*., 2005c).

Substrate is another factor influencing competition between Accumulibacter and GAOs. Oehmen and colleagues (2005a) found that the dominant GAOs were Competibacter and *Alphaproteobacteria* when fed with acetate and propionate respectively. Further, Accumulibacter was able to immediately switch between acetate and propionate as a substrate with no obvious preference, and metabolize them at similar rates (Oehmen *et al*., 2005b). In contrast, both GAO groups took up the substrate at a slower rate after being switched from a previous substrate, exhibiting some substrate preference (Dai *et al*., 2007). Based on these physiological differences, a feeding strategy was developed by alternating acetate and propionate under pH 7–8 to eliminate both GAO groups. Under these conditions, Accumulibacter was enriched to > 90% of total bacteria (Lu *et al*., 2006), providing a near-'pure' sludge to study Accumulibacter metabolism more accurately.

Temperature, as another important driving force to shape the competition, was shown to be optimal at 30°C for Competibacter for both anaerobic and aerobic metabolism (Lopez-Vazquez *et al*., 2007; 2008b). Under longer-term operation at 10°C, a shift in the dominant bacterial population to Accumulibacter occurred, likely due to decreased rates of acetate uptake and biomass production by Competibacter (Lopez-Vazquez *et al*., 2009a).

To evaluate the combined effects of pH, carbon substrate and temperature, Lopez-Vazquez and colleagues (2009b) developed a metabolic model with these factors incorporated to describe the PAO–GAO competition. Based on the model, they estimated that at 10°C Accumulibacter dominated over both Competibacter and *Alphaproteobacteria* GAOs, regardless of carbon substrate and operating pH; at 20°C, either a combined acetate/propionate feeding or a high operating pH (e.g. 7.5) was sufficient to maintain the dominance of Accumulibacter; and when temperature increased to 30°C, both the combined acetate/propionate feeding and a high operating pH were required to minimize GAOs.

An interesting question was raised regarding the ability of Accumulibacter to use the glycogen accumulating metabolism, effectively functioning as a GAO. Zhou and colleagues (2008) observed that a polyP-depleted Accumulibacter-enriched sludge was still able to take up acetate and form PHA, with substantially increased glycogen degradation in the absence of P cycling, therefore exhibiting some GAO phenotypes. This contradicts an earlier similar study that showed glycogen could not be used as a sole energy source to take up acetate (Brdjanovic *et al*., 1998). After a careful comparison between these two studies, Lopez-Vazquez and colleagues (2008a) found the main differences were the carbon substrate (alternating acetate/propionate versus acetate) and pH (7–8 versus 7.0). They postulated that different substrates may have enriched different Accumulibacter strains with metabolic variation, such as in glycolytic pathways. This might also explain previous reports of the ED pathway activity (Hesselmann *et al*., 2000), even though this pathway components are missing in the currently available Accumulibacter genome (Garcia Martin *et al*., 2006)

Ecophysiological differentiation, population structure and dynamics

Besides the postulated metabolic variation in glycogen utilization mentioned above, other physiological variations within Accumulibacter have also been observed. For example, not all Accumulibacter cells in full-scale sludges were accumulating polyP *in situ* (Zilles *et al*., 2002a; Kong *et al*., 2004; Wong *et al*., 2005), and the physiological

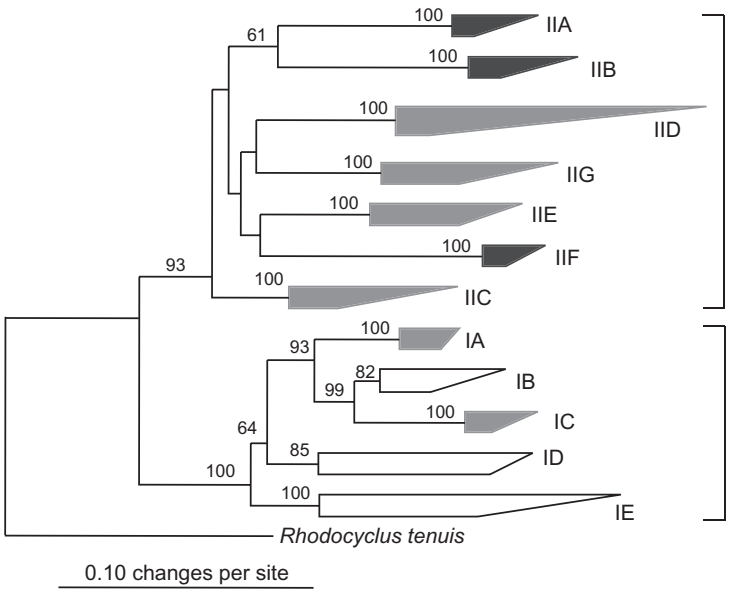

Fig. 3. Maximum likelihood tree of *ppk1* genes (McMahon *et al*., 2002; 2007; He *et al*., 2007; Peterson *et al*., 2008) from the Accumulibacter lineage. A *ppk1* alignment with positions of < 50% maximum frequency masked was used to construct the tree using GARLI with a general time-reversible model and Gamma-distributed among-site rate heterogeneity to find the best tree and RAxML for 100 bootstrap resamplings. The Accumulibacter lineage was comprised of two Types (I and II indicated by brackets), each consisting of a number of monophyletic clades. Metagenome sequences were derived from sludges enriched in clade IIA. Clades in black were exclusively derived from EBPR systems and environmental samples collected near EBPR treatment plants. Clades in grey contained sequences from both EBPR systems and natural habitats not associated with EBPR treatment plants. Clades in white exclusively consisted of environmental samples, particularly from an estuary sediment.

heterogeneity is best demonstrated in the case of nitrate reduction.

Accumulibacter was the most abundant bacterial group responsible for denitrifying P removal in an anaerobic/anoxic reactor supplied with nitrate, and was able to switch from anoxic to aerobic mode immediately, while a 5 h lag occurred upon switching from the other way around (Zeng *et al*., 2003a). However, the currently available Accumulibacter genome lacks the respiratory nitrate reductase (NAR), even though it possesses genes for downstream denitrification (Garcia Martin *et al*., 2006). This contradiction was consistent with the observation that some, but not all, Accumulibacter could take up P when nitrate was supplied as the sole electron acceptor in full-scale sludges (Kong *et al*., 2004). Striking evidence was also provided by Carvalho and colleagues (2007). They operated two lab-scale reactors fed with acetate and propionate respectively. Two distinct morphologies were observed for Accumulibacter. The coccus-morphotype dominated the acetate-fed reactor and was unable to use nitrate as an electron acceptor, whereas the rod-morphotype was highly enriched in the propionate-fed reactor, capable of using both nitrate and nitrite. This interesting observation was further supported by a study, where a nitrite-adapted sludge, dominated by coccus-Accumulibacter, was unable to reduce nitrate, and had to rely on the flanking community to produce nitrite for P uptake, based on both short- and long-term nitrate dosage experiments (Guisasola *et al*., 2009). These studies indicate that the observed phenotypic variation was derived from different populations within Accumulibacter, rather than from different physiological states of the same population.

Since PAOmix FISH probe-defined Accumulibacter is a not a physiologically homogenous group, developing tools to distinguish the subgroups (i.e. Types or clades) would be beneficial to study Accumulibacter ecology and explain the conflicting findings. The most frequently used phylogenetic marker, the 16S rRNA gene, cannot provide enough resolution for this purpose, because even 16S rRNA sequences retrieved from geographically and operationally distinct EBPR systems are highly conserved among Accumulibacter (He *et al*., 2007). Other genetic markers with more sequence variation have been used to reveal finer-scale differences within this group, including the 16S–23S rRNA internal transcribed spacer (ITS) region (He *et al*., 2006; 2010b), and genes encoding nitrite reductase (*nirS*) (Miyauchi *et al*., 2007), polyphosphate kinase 1 (*ppk1*) (He *et al*., 2007) and PHA synthase (*phaC*) (Wang *et al*., 2008). So far, *ppk1* has been the most extensively examined.

ppk1 is a single-copy gene in Accumulibacter and appears to evolve four times faster than 16S rRNA genes (Kunin *et al*., 2008), making it a good phylogenetic marker to observe fine-scale differences. Motivated by the potential role of this gene in polyP accumulation, McMahon and colleagues (2002) retrieved *ppk1* from Accumulibacter in lab-scale reactors and detected its expression. From an extensive census of bacterial *ppk1* genes from a number of full-scale EBPR and non-EBPR sludges, McMahon and colleagues (2007) observed two major Types of Accumulibacter (I and II respectively) (Fig. 3), and designed a *ppk1* primer set targeting total Accumulibacter. Using this primer set, He and colleagues (2007) retrieved fragments of Accumulibacter *ppk1* genes from a few more EBPR facilities, and found phylogenies reconstructed using 16S rRNA and *ppk1* genes were largely congruent. Moreover, the *ppk1* phylogeny exhibited a higher resolution, with at least five subgroups (clades) emerging under the two

major Types, suggesting the Accumulibacter lineage was more diverse than previously recognized. The distribution patterns of these clades suggested that Accumulibacter population structure varied among different EBPR systems and also changed temporally within a system. The authors further hypothesized that these Accumulibacter clades were ecologically distinct.

As anticipated, *ppk1*-defined Types/clades are useful in explaining some discrepancies in the literature. For example, the NAR-lacking dominant Accumulibacter strain during the metagenomic analysis was affiliated with later defined clade IIA. By performing a series of batch tests on two lab-scale sludges enriched in clades IA and IIA, respectively, Flowers and colleagues (2009) showed that clade IA-enriched sludge was able to couple nitrate reduction with P uptake, but clade IIA-enriched sludge could not, agreeing with predictions based on the clade IIA genome. Using the 16S rRNA FISH probes designed by Flowers and colleagues (2009) that differentiate Types I and II, Oehmen and colleagues (2010a) were able to relate the nitrate-reducing and non-nitrate-reducing Accumulibacter subgroups observed by Carvalho and colleagues (2007) to Types I and II respectively. Further, incorporating this metabolic difference into modelling enabled a successful prediction of the abundances of these Accumulibacter subgroups in EBPR sludges (Oehmen *et al*., 2010b). As lab-scale reactors only enrich for a subset of the Accumulibacter lineage (He *et al*., 2007), whether this denitrification difference can be generalized to all clades within each Type needs further investigation. It should be noted that the Type I/II probes designed by Flowers and colleagues (2009) cannot inclusively target all members within each Type, and particularly miss some sequences that were exclusively present in full-scale treatment plants. Therefore, when using these two probes, FISH with the PAOmix probes should be applied to check if the sum from Type I/II probes can account for the total Accumulibacter, especially for full-scale sludges.

The existence of multiple distinct Types/clades may also partly explain some discrepancies among the post-genomics investigations of Accumulibacter gene expression and metabolic regulation, and provide insight on the debate concerning the anaerobic operation of the TCA cycle. For example, Wilmes and colleagues (2008b) found that proteins with amino acid variation were more abundant than their orthologues in the available clade IIA genome, indicating genetic diversity among closely related Accumulibacter. Indeed, they identified clades IA and IID as the dominant Accumulibacter in their lab-scale system, in contrast with the clade IIA-enriched sludges used for the metagenomics (Garcia Martin *et al*., 2006) and gene expression analyses by microarrays (He *et al*., 2010a) and qRT-PCR (He and McMahon, 2011). As mentioned earlier, the variation in metabolic regulation was more evidently shown by radiolabelled metaproteomics revealing the different anaerobic involvements of the TCA cycle by two sludges (Wexler *et al*., 2009). The sludge exhibiting enhanced anaerobic TCA cycle expression was found to consist of clades IC, IIA, IIB, IIC and IID, while the other one was abundant in clade IA, suggesting these clades differ in anaerobic TCA cycle operation, which might explain the controversy surrounding this topic in the literature.

Whether or not the published contradictions regarding the EMP (Mino *et al*., 1987; Garcia Martin *et al*., 2006) versus ED (Maurer *et al*., 1997; Hesselmann *et al*., 2000) glycolytic pathways can also be attributed to strain variation remains to be investigated. Accumulibacter is probably versatile in metabolism as suggested by its relatively large genome (e.g. 5.31 Mbp for clade IIA, compared with the average of 3.67 Mbp from all 1092 finished bacterial genomes currently available in the IMG database). The metabolic versatility and diversity can be illustrated by the possession of genes enabling all three operating modes of the TCA cycle, and is also evident by occurrences of Accumulibacter in different EBPR systems and natural aquatic environments (Peterson *et al*., 2008). Therefore, we expect some metabolic differences may be due to the metabolic versatility, while some can be directly linked to genotypic variation (e.g. nitrate reduction). To which extent these subgroups differ and which of their physiological differences can be attributed to metabolic versatility and genotypic variation, respectively, remain to be elucidated. As such, clade-level quantification of populations based on tools, such as quantitative PCR (He *et al*., 2007) or extensive sequencing of protein-coding phylogenetic markers (e.g. *ppk1*), is highly recommended for future studies to avoid seemingly conflicting results and to reveal more of the important variations within Accumulibacter. These intra-population differences can then be incorporated into metabolic modelling to enable accurate prediction of these subgroups (Oehmen *et al*., 2010b,c).

The dynamics of Accumulibacter population structure was studied in the context of total bacteria by using community fingerprint techniques, such as automated ribosomal intergenic spacer analysis (ARISA) of the 16S–23S rRNA ITS region (He *et al*., 2010b) and terminal-restriction fragment length polymorphism (T-RFLP) analyses of 16S rRNA and *ppk1* genes (Slater *et al*., 2010). He and colleagues (2010b) investigated two lab-scale reactors operated under identical conditions in parallel for 6 months, and found the distribution of the two detected clades (IA and IIA) within the total Accumulibacter was quite stable in one reactor, while comparatively dynamic in the other reactor. Similarly, Slater and colleagues (2010) also observed a divergence in microbial community and Accumulibacter population structure during a 35-day operation of four replicate mini-reactors. In both studies, good EBPR activity was positively associated with the abun-

dance of total Accumulibacter. In addition, He and colleagues (2010b) found that the variance in the distribution of clades IA and IIA did not appear to affect reactor performance therefore suggesting functional redundancy of these two Accumulibacter clades. Slater and colleagues (2010) noticed that clade IA was associated with good EBPR performance while IIC with poor performance where Competibacter GAOs were abundant. Therefore, Slater and colleagues (2010) suspected that IIC may have a higher substrate affinity than IA, which allows IIC to be selected for under increased competition for the substrate from increased GAOs for the substrate. To date, what governs the observed dynamics of Accumulibacter population structure remains largely unknown.

In summary, although sequence variation, metabolic and physiological differences have been observed among Accumulibacter, further research is needed to unambiguously link phylogenic identities to microbial functions and ecological niches. In general, lab-scale reactors exhibited a lower Accumulibacter diversity than full-scale systems, which presumably harbour more ecological niches (He *et al*., 2007). Some of the niches may correspond to the physiological variations of different members within the Accumulibacter lineage, such as in the cases of nitrate reduction (Carvalho *et al*., 2007; Flowers *et al*., 2009), substrate preference towards acetate or propionate (Carvalho *et al*., 2007), metabolic regulation (Wexler *et al*., 2009) and substrate affinity (Slater *et al*., 2010). Therefore, the diversity may provide some functional redundancy and a higher system resiliency in full-scale systems where the disturbance in niche partitioning occurs frequently. Closely monitored population dynamics associated with system performance in full-scale treatment plants will be helpful to understand the ecological niches and distribution of these Accumulibacter subgroups.

Ecological distribution in EBPR systems

Accumulibacter was originally identified in acetate-fed lab-scale reactors (Hesselmann *et al*., 1999; Crocetti *et al*., 2000), and was later repetitively identified in such systems, often ranging from 40% to 80% of total bacteria. Pijuan and colleagues (2004) were the first to report Accumulibacter as dominant PAOs in propionate-fed reactors, in which they accounted for ~55% of total bacteria. Higher enrichment (up to 89%) with propionate feed was maintained by Carvalho and colleagues (2007). By alternating acetate and propionate feeds routinely, Lu and colleagues (2006) could achieve > 90% enrichment.

Since the first confirmation of Accumulibacter abundance and role in full-scale treatment plants (Zilles *et al*., 2002a), a number of studies have been conducted in treatment plants around the world to study factors influencing the distribution of this lineage and its correlation to EBPR performance. These systems varied in configurations, operational conditions, wastewater composition, climate and discharge standards. In the USA, a survey of five EBPR facilities showed Accumulibacter abundances of 9–24% of total cells, and > 80% of Accumulibacter were estimated to have high polyP contents (He *et al*., 2008). Notably, unlike many other studies where the original PAOmix probes were used, the probes applied in this study were PAOmix with several degenerate bases and included RHC439, a probe also targeting some bacteria in the '*Dechloromonas agitatus* subgroup' within the *Rhodocyclaceae* family (Zilles *et al*., 2002a). Another study conducted in the USA estimated Accumulibacter constituted 5–20% of total activated sludge bacteria, and showed the process performance and stability was positively correlated to the influent readily biodegradable COD (chemical oxygen demand) to P ratios (Gu *et al*., 2008). In Denmark, 10 EBPR plants were investigated by Kong and colleagues (2005) and they observed higher Accumulibacter abundances (9–17%) in facilities primarily treating domestic wastewater, while much lower abundances (< 3%) in most industrial treatment plants where wastewater had higher amino acid contents, with Actinobacteria as the dominant PAOs. This is consistent with the carbon substrate specificity determined by using FISH-MAR (Kong *et al*., 2004), therefore suggesting wastewater organic carbon composition as a key factor influencing PAO population assembly. In the Netherlands, Accumulibacter abundance (5.7–16.4% in seven EBPR plants) was correlated to a well-defined denitrification stage and high pHs in the anaerobic zone (López-Vázquez *et al*., 2008). In Australia, Accumulibacter were present at 7–12% of total bacteria in four facilities examined by Saunders and colleagues (2003), and 5–10% in two facilities studied by Pijuan and colleagues (2008). However, another survey conducted on nine treatments plants in the eastern states of Australia showed that Accumulibacter were rare in most facilities, and instead Actinobacteria may have been the primary PAOs (Beer *et al*., 2006). A similar controversy also occurred in treatment plants surveyed in Japan. A study of EBPR facilities mainly treating municipal wastewater in Tokyo showed that Accumulibacter accounted for 7–17% of total bacteria and their polyP accumulation was confirmed by dual labelling with FISH and polyP staining (Chua *et al*., 2006). However, Wong and colleagues (2005) tested another nine treatment facilities (eight in Tokyo and one in Kawasaki), most of which mainly treated domestic wastewater, and found that although Accumulibacter was > 10% of total bacteria in most EBPR systems, a large fraction of Accumulibacter contained little or no polyP.

Although DAPI (4′,6-diamidino-2-phenylindole) staining has been a popular method to detect cells with high polyP contents and was applied in some of the studies dis-

cussed above (e.g. Zilles *et al*., 2002a; Wong *et al*., 2005; Beer *et al*., 2006; Gu *et al*., 2008; He *et al*., 2008), the specificity of DAPI towards polyP has not been fully proven and it is difficult to quantify polyP contents based on the staining signal intensity. In addition, it was noted that the FISH procedures interfered with the polyP staining by DAPI (Zilles *et al*., 2002b). Therefore, researchers have to conduct DAPI staining and FISH sequentially and colocalize the microscopic slide to the same spot for dual labelling (Liu *et al*., 2001; Wong *et al*., 2005), or mathematically solve the proportion of FISH-positive cells with high polyP contents based on the enrichment factors for FISH- and polyP-positive cells, respectively, after a physical PAO-enrichment procedure (Zilles *et al*., 2002a; He *et al*., 2008). Recently, a Raman microscopy was applied to an Accumulibacter-enriched sludge to provide a relative quantification of polyP at an individual cell level and heterogeneity of polyP levels within the population was revealed (Majed *et al*., 2009). This technique was further successfully applied to quantify PHA and glycogen, simultaneously with polyP (Majed and Gu, 2010). Therefore, when combined with FISH, Raman microscopy can link the PAO phylogenetic identities to their biopolymer transformation functions. However whether the Raman spectra for these polymers are conserved in other PAOs requires further investigation.

Besides in traditional anaerobic/aerobic EBPR systems, Accumulibacter were also the dominant PAOs in anaerobic/anoxic (Zeng *et al*., 2003b) and continuously aerated reactors (Ahn *et al*., 2007; Vargas *et al*., 2009) discussed earlier. Recently, novel configurations were designed to simultaneously achieve nitrification, denitrification and P removal (Zeng *et al*., 2003a), such as granular sludge (Meyer *et al*., 2005; Lemaire *et al*., 2008; Yilmaz *et al*., 2008). This configuration takes the advantage of stratified sludge granules, in which oxygen penetrates the outer layer leaving anoxic zones in the centre where denitrification occurs. Accumulibacter dominated the outermost layer, responsible for P removal, and Competibacter dominated the central zone, accounting for denitrification (Lemaire *et al*., 2008). However, Competibacter were eliminated and Accumulibacter became the major contributor to denitrification when granular sludge was exposed to real wastewater, likely due to the end nitrification product shifting from nitrate to nitrite by elimination of nitrite oxidizers (Yilmaz *et al*., 2008).

It should be noted that the boundary of the Accumulibacter lineage is vague in phylogenetic trees, because of the limited number of bacterial isolates representing the *Rhodocyclaceae* family. Phylogenetically, *Dechloromonas* spp. are very close to Accumulibacter. Sequences retrieved from EBPR systems were sometimes affiliated with *Dechloromonas* spp., such as in aerated-anoxic Orbal® processes (Zilles *et al*., 2002a). Indeed, when Goel and colleagues (2005) operated a lab-scale reactor simulating the Orbal® process by introducing low levels of DO in the anaerobic phase, *Dechloromonas*-like bacteria were enriched as primary PAOs. In addition, a study of microbial community structure and function in a full-scale EBPR sludge by combining a full-cycle rRNA approach, chemical staining and microautoradiography revealed that bacteria affiliated with *Dechloromonas* spp. were able to take up short-chain fatty acids, accumulate PHA and polyP, thus exhibiting the PAO phenotype (Kong *et al*., 2007). However, the GAO phenotype was also observed from *Dechloromonas*-related bacteria in a continuously aerated lab-scale EBPR reactor (Ahn *et al*., 2007). It is likely that some *Dechloromonas*-like bacteria are also involved in P removal under some conditions, since they are close relatives of Accumulibacter, and thus may share some physiological characteristics. Alternatively, some *Dechloromonas*-like sequences probably were actually from a subgroup of Accumulibacter that could not be resolved due to the vague boundary of this lineage.

In summary, these studies confirmed the abundance and role of Accumulibacter in P removal in lab-scale and some full-scale EBPR systems with varied configurations. They also clearly indicated a variation in polyP-accumulating capabilities within Accumulibacter and suggested the importance of other PAOs in full-scale systems with a higher degree of niche heterogeneity. For example, VFAs, the main carbon sources in lab-scale reactors, account for only a fraction of hydrolysis products from total organic matter in real wastewater. Accumulibacter's preference towards VFAs may have prevented their dominance in systems with higher contents of other organic substrates (Nielsen *et al*., 2010). Furthermore, other differences between these two scales of EBPR systems also exist. For example, VFAs are generated from hydrolysis of complex carbons in the anaerobic zone in many treatment plants and thus their concentrations may be maintained at low levels due to simultaneous microbial utilization, contrasting to the pulse dosage in most lab-scale reactors. These differences may limit a generalization of knowledge gained from lab-scale to full-scale systems, such as in the case of PHA accumulation, one of the criteria in PAO definition previously used by many researchers. Other PAOs with varied phenotypes occupying different niches, particularly the Actinobacteria-related PAOs, need more research attention, since their preferred substrates, amino acids, constitute a large fraction of total organic carbons and they were prominent in many treatment plants (Nielsen *et al*., 2010).

Environmental distribution

Although members of the Accumulibacter lineage appear to be particularly well adapted to living in organic carbon-

and nutrient-rich activated sludge systems, they likely evolved to thrive in organic carbon- and nutrient-limited natural aquatic habitats such as lakes and rivers. This is inferred form the presence of genes for carbon and nitrogen fixation in the Accumulibacter clade IIA genome, as well as those encoding apparatuses for high-affinity P_i transport and flagella biosynthesis (Garcia Martin *et al*., 2006). In addition, a high degree of genome-level sequence conservation was found between the Accumulibacter strains sequenced in US and OZ sludges. This suggested that Accumulibacter dispersal rates are rapid as compared with rates of sequence change and hinted at the importance of environmental reservoirs for Accumulibacter outside of wastewater treatment systems. Although multiple strains were present in each reactor, no evidence for recombination among them was found, and Kunin and colleagues (2008) suggested this apparent asexuality should lead to local differentiation. However, phylogenies constructed for the dominant species sequenced in each sludge using single-copy genes provided no evidence for geographic isolation, and instead supported the hypothesis of global dispersal (Kunin *et al*., 2008). These findings prompted a hunt for Accumulibacter in natural aquatic habitats using *ppk1* as a phylogenetic marker (Peterson *et al*., 2008). A remarkable diversity and phylogenetic structure within this lineage was discovered in freshwater sediments, lakes, streams and an estuary (Fig. 3). The two Types defined by McMahon and colleagues (2002; 2007) and the clades identified by He and colleagues (2007) were robust to the addition of more than 700 new *ppk1* sequences to the phylogeny, although a total of 12 coherent clades are now apparent (Peterson *et al*., 2008). These clades were unevenly distributed among the sampled habitats, and were statistically underdispersed across the entire data set as determined using novel phylogenetic ecology tests for community structure. This pattern could be the result of strong habitat filtering, suggesting that unique subclades of Accumulibacter are ecologically differentiated and sorted into appropriate habitats. The extent to which gene flow among populations in natural habitats and those in wastewater treatment plants contributes to diversification of Accumulibacter is an exciting potential research question, particularly given the metagenomic sequencing results that suggest gene flow among co-occurring strains is minimal (Kunin *et al*., 2008).

Dispersal and ecological differentiation are likely not the only forces structuring Accumulibacter lineages within and across habitats. Further analysis of the metagenomic sequences obtained from the US and OZ sludges revealed regions of markedly higher variation associated with CRISPR elements (Kunin *et al*., 2008). CRISPRs are rapidly evolving clusters of short repeats with regularly interspersed unique 'spacer' sequences that are often derived from phages, probably conferring immunity to phages (Barrangou *et al*., 2007). Kunin and colleagues (2008) hypothesized that Accumulibacter strains are globally dispersed but must adapt locally to persistent phage predation pressure. Microarray analysis of cDNA generated from the US sludge provided further evidence for the presence of phage, since many phage-related gene transcripts were detected during normal bioreactor operation (Kunin *et al*., 2008; He *et al*., 2010a). The role of phages in maintaining EBPR community structure and causing Accumulibacter population dynamics is unknown, but will surely be an area of fruitful future research. Recently, a phage attack was hypothesized to have caused the declination in P removal capacity of a lab-scale reactor due to the microscopic observation of lysed Accumulibacter cells and a sharp decrease in Accumulibacter abundance, concomitant with the detection of a very high abundance of phage tail sheath proteins (Barr *et al*., 2010). This hypothesis was further supported by deliberate infections of EBPR sludges with phage-containing supernatant collected from the previously failed reactor. Deterioration in P removal, associated with decrease in Accumulibacter and increase in phage abundances, was observed after the infections (Barr *et al*., 2010). Although the evidence was largely indirect, it surely promotes the interest to investigate phage dynamics and activity as a new research dimension for EBPR system stability.

Another intriguing question surrounds the natural occurrences of Accumulibacter. The detection in lake sediments coincides with the fact that some lake sediments also experience anaerobic and aerobic cycles due to seasonal mixing, together with changes in bioavailability and speciation of P in the water column and sediments. For example, polyp-accumulating organisms were found in aquatic sediments (Hupfer *et al*., 2007). The presence of Accumulibacter in such environments stimulates our curiosity about their *in situ* physiology and contribution to P cycling in natural aquatic environments. Nevertheless, their abundance in such habitats was much lower than that in EBPR systems (Peterson *et al*., 2008).

Concluding remarks

In the past 6 years, with new powerful molecular tools to investigate EBPR microbiology, much fruitful research has greatly advanced our understanding of Accumulibacter metabolism, physiology, fine-scale population structure, ecophysiological differentiation, and distribution in EBPR systems and natural environments. Some long-existing puzzles were resolved and new insights were gained. However, most of our understanding has been generated from lab-scale reactors containing 'domesticated' sludges: fed with simple model carbon sources (acetate and/or propionate) and operated under relatively constant and optimal conditions. More research effort needs to be

directed towards full-scale systems containing 'real' sludges: with complex influent composition and dynamic operating conditions, especially when multiple processes may be at play. Various high-throughput tools are becoming more affordable and applicable today, facilitating microbial analyses at the community level, such as metagenomics, metatranscriptomics, metaproteomics and metabolomics, and at the single-cell level by sequencing the genome of a single cell following cell sorting and screening based on phylogenic identity or functions the cell performs. Molecular systems biology approaches will help to gain a deeper understanding of Accumulibacter metabolic regulation under different conditions, its interactions with other microbial populations and the functions of the community as a whole in complex systems. More secrets will surely be revealed about this interesting and once-called microbial 'black box'.

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