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REVIEW

The Microbiology of Phosphorus Removal in Activated Sludge Processes- the Current State of Play

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This review discusses critically what we know and would like to know about the microbiology of phosphorus (P) removal in activated sludge systems. In particular, the description of the genome sequences of two strains of the polyphosphate accumulating organism found in these processes, *Candidatus* 'Accumulibacter phosphatis', allows us to address many of the previously unanswered questions relating to how these processes behave, and to raise new questions about the microbiology of P removal. This article attempts to be deliberately speculative, and inevitably subjective, but hopefully at the same time useful to those who have an active interest in these environmentally very important processes.

***Keywords*:** enhanced biological phosphorus removal, *Accumulibacter*, *Defluviicoccus*, *Competibacter*

What do we know now about the process of phosphorus removal?

Like many other wastewater treatment processes, our knowledge of the engineering/operational features of enhanced biological phosphorus removal (EBPR) activated sludge processes predated our understanding of its microbiology (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). It has been agreed generally that successful P removal requires the biomass to be cycled continuously through anaerobic and aerobic operating zones, conditions thought necessary for the enrichment and eventual domination of the phosphate accumulating organisms or PAO (polyphosphate accumulating organism) populations (Mino *et al.*, 1998; Seviour *et al.*, 2003; Oehmen *et al.*, 2007). These assimilate substrates anaerobically and synthesize poly ß hydroxyalkanoates (PHA), using stored polyphosphate (polyP) as the energy source and glycogen as source of energy and reducing power. Under subsequent aerobic conditions the stored PHA is then respired aerobically by the PAO, and the energy used to assimilate phosphate, to synthesize polyP and with the available stored carbon, to replenish their glycogen stores. As exogenous readily metabolizable substrates are no longer available aerobically, populations able to use their stored PHA are selectively advantaged and become dominant (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). One would expect organisms able to cope with these changing and stressful conditions to have unusual and fascinating properties.

It is now clear that EBPR can also occur under continuously aerated 'aerobic' conditions where the addition of the phosphorus and the carbon source (acetate) are temporarily separated and not added simultaneously (Ahn *et al.*, 2007), as happens with anaerobic;aerobic EBPR systems. EBPR can also take place anaerobically in the presence of nitrate, as long as no readily biodegradable substrates are present (Seviour *et al.*, 2003; Barnard and Abraham, 2006; Oehmen *et al.*, 2007). In denitrifying EBPR systems PAO cells use nitrite (not nitrate? - but see later) and not oxygen as the electron acceptor, and P assimilation and polyP synthesis occur by populations called denitrifying PAO or DPAO (Seviour *et al.*, 2003). The attractions of denitrifying EBPR processes are clear. Achieving simultaneously nitrogen (N) and P removal with less sludge production and no requirement for aeration makes such systems more economical (Kuba *et al.*, 1996; Carvalho *et al.*, 2007).

What do we know about the microbes responsible for EBPR?

The application of molecular culture independent methods, based largely on SSU rRNA sequence analyses to EBPR communities has radically changed our views about the likely identity of the PAO in the past decade (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). Whereas early culture dependent methods suggested the gammaproteobacterial *Acinetobacter* spp. were important, none of those cultured were ever shown to possess the supposed phenotype of a PAO, raising doubts as to their involvement in EBPR. Clone library analyses followed by quantitative fluorescence *in situ* hybridization (FISH) confirmed this view (Wagner *et al.*, 1993; Wagner *et al.*, 1994), and instead indicated that *Rhodocyclus*-related *Betaproteobacteria*, given the name *Candidatus* 'Accumulibacter phosphatis' were more likely candidates (Hesselmann *et al.*, 1999). However, not all clone libraries from EBPR proc-

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esses have detected these populations, possibly because of problems in extracting the DNA from such heavily capsulated clustered cells. An *in situ* phenotype expected of a PAO has since been demonstrated frequently for them (Crocetti *et al.*, 2000; Kong *et al.*, 2001; Kong *et al.*, 2004) in both lab scale and full scale EBPR systems. The availability now of the genome sequence of *Candidatus* 'Aumulibacter phosphatis' from a heavily enriched community (García Martín *et al.*, 2006; Lu *et al.*, 2006) has already had a considerable impact on our understanding of these PAO populations, as this review will hopefully illustrate.

It seems clear now that *Accumulibacter* are not the only PAO populations in EBPR systems. Many FISH based studies have detected polyP by DAPI staining in other unrelated organisms in these communities (Liu *et al.*, 2000; Wong *et al.*, 2005; Beer *et al.*, 2006; He *et al.*, 2008). In fact evidence shows the *Accumulibacter*/*Beta-Proteobacteria* often make up only a relatively small proportion of cells accumulating polyP, especially in full scale plants. So that raises the important issue of what is the identity of all the other PAO, a question which needs to be answered before we can feel confident we understand EBPR. At the moment we don't really have an answer, and so whether recently cultured polyP accumulating organisms like *Malikia granosa* (Spring *et al.*, 2005), *Stenotrophomonas* (Ghosh *et al.*, 2005) or *Gemmatimonas aurantiaca* (Zhang *et al.*, 2003) are shown eventually to play an important role in these systems remains to be seen.

We do know that many full scale EBPR plant communities contain high numbers of polyP staining and as-yet uncultured *Tetrasphaera*-related *Actinobacteria* (e.g., Beer *et al.*, 2006; e.g., Kong *et al.*, 2007). Kong *et al.* (2005) demonstrated that their *in situ* physiology was not that expected of conventional PAO, in that they did not assimilate acetate or synthesise PHA anaerobically, as the *Accumulibacter* do. Instead they appear to utilize amino acids, but in what form these are stored is unclear. However, these *Actinobacteria* have a phenotype different to that normally used to 'define' what we understand a PAO to be, and as a result we believe it is appropriate to use a wider and looser definition of a PAO. In our view, any population which accumulates more P than it requires for growth and which stains positively for polyP should be considered as a putative PAO, regardless of whether it synthesizes PHA anaerobically. As Kong *et al.* (2005) showed, other survival strategies exist to allow cells to cope with the feast: famine conditions of EBPR anaerobic:aerobic recycling, and more are likely to emerge as our understanding of EBPR microbiology increases. Certainly microautoradiography (MAR) studies invariably reveal that anaerobic acetate uptake in EBPR plants is a feature of many cells other than *Accumulibacter* (Kong *et al.*, 2004), but not all these stain positively for PHA with nile blue stain. Alternatively, sufficient metabolizable substrates may still be available in the aerobic zone to allow some populations not storing PHA to scavenge these and assimilate P into polyP.

While some molecular data have suggested that the PAO populations active under anoxic and aerobic conditions might be different (Ahn *et al.*, 2002; Lee *et al.*, 2003), it seems increasingly likely that *Accumulibacter* are the major PAO populations in both denitrifying and conventional EBPR processes (Zeng *et al.*, 2003; Kong *et al.*, 2004; Carvalho *et al.*, 2007), emphasizing their metabolic versatility. Yet the story may be more complex than that. Some evidence implies that different *Accumulibacter* strains with different physiologies may exist. Thus, Carvalho *et al.* (2007) suggested (albeit from indirect evidence) that rod shaped *Accumulibacter* used nitrate, nitrite and oxygen as electron acceptors for EBPR, whereas the more common *Accumulibacter* morphotype of large coccal cells used nitrite and oxygen. Resolving any such ecophysiological variations among *Accumulibacter* PAO strains is vitally important in our attempts to monitor EBPR processes better, and their possible phylogenetic diversity will be discussed in more detail next.

Are all *Accumulibacter* PAO the same?

It is becoming clear that considerable phylogenetic diversity probably exists among the *Accumulibacter* PAO closely related to *Rhodocyclus* in the *Betaproteobacteria*, but whether this is associated with corresponding physiological and ecological diversity is not so apparent. Understanding which factors might determine which populations are present in a plant, how these might impact on EBPR capacity and the factors responsible for their selection are questions in need of urgent attention.

This group of organisms was first suggested as PAO by Hesselmann *et al.* (1999) and confirmed by Crocetti *et al.* (2000) who described a set of probes designed from their clone library data to target the 16S rRNA of its members, which were 98% similar to each other. Zilles *et al.* (2002) then redesigned their probes to include two *Dechloromonas* related sequences. An assessment by Saunders (2005) suggested that the original probes of Crocetti *et al.* (2000) are the most sensitive and specific for *Accumulibacter* PAO. Some studies have cast doubt over the value of 16S rRNA gene analysis in revealing the full phylogenetic diversity of the *Accumulibacter* PAO. A similarity level of >97% is used frequently to delineate individual species (Stackebrandt and Goebel, 1994), although many believe this should be increased to >98.7~99% (Stackebrandt and Ebers, 2006).

The existence of *Accumulibacter* DPAOs (see above), morphological differences between them and cells responding to the PAO FISH probes which do not accumulate polyP (Kong *et al.*, 2004) would all suggest the existence of phenotypically distinct strains (He *et al.*, 2007). For example, as discussed above, Carvalho *et al.* (2007) suggested that one of the two morphologically distinct *Accumulibacter* PAO they saw with the PAO FISH probes use nitrate as an electron acceptor, despite the absence of the nitrate reductase gene required for this in the whole genome sequence of the '*Accumulibacter phosphatis*' strains in enriched lab-scale anaerobic:aerobic EBPR processes fed acetate or propionate (García Martín *et al.*, 2006). Furthermore, other *Accumulibacter* PAO in both communities showed 15% genome sequence divergence from the dominant strains, likely to translate into phenotypic differences (Carvalho *et al.*, 2007). By comparative analysis of the internally transcribed spacer region (ITS) in the *rrn* operon, He *et al.* (2006) revealed much finer scale differences between members of the *Accumulibacter* PAO than were apparent with 16S rRNA. McMahon *et al.* (2007) have since suggested using the gene

encoding the polyphosphate kinase (ppk) involved in the production of polyphosphate, as a marker capable of elucidating *Accumulibacter* biodiversity. They showed that phylogenetic trees generated with *ppk* and 16S rRNA genes were largely congruent (He *et al.*, 2007). However, they could group strains by *ppk* sequence information into five distinct clades designated I, IIA, IIB, IIC, and IID (He *et al.*, 2007). The additional phylogenetic resolution provided by the *ppk* sequences allowed the design of appropriate primers sets to differentiate between members of each clade. Plant surveys based on this gene marker showed their presence varied depending on plant configuration and location, information not forthcoming from 16S rRNA based analyses. Thus, in lab scale reactors fed synthetic sewage, only subgroups I and IIA were present, while in full scale plants members of at least three of the five clades were present. The suggestion was made that *ppk* diversity was related to the 'complexity' of the system, with lab scale reactors operating under controlled conditions and fed a simple defined carbon source exhibiting the lowest diversity. Application of this approach to more plants from different countries and operating under different conditions is needed before the relevance of this information to EBPR performance can be assessed. Whether members of individual clades are associated with plant characteristics, and can be used for monitoring is an intriguing prospect. At the moment the ecophysiological importance of *ppk* sequence variations in *Accumulibacter* on process performance is not known. Whether similar studies with other key genes involved in EBPR in *Accumulibacter,* for example those encoding for phaC synthases, show additional diversity remains to be seen.

Why do EBPR processes fail?

The general experience is that full scale EBPR processes dealing with complex and variable feeds are especially notoriously unreliable, and in many countries, chemical P removal is used as a back-up to finally polish their effluents (Seviour *et al.*, 2003). There may be several sound explanations for poor EBPR capacity, and not all of them microbiological (Seviour *et al.*, 2003; Barnard and Abraham, 2006). Increasingly attention is being directed to the possible negative impact on EBPR of bacterial populations known generically as the glycogen accumulating organisms (GAO) (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). Their anaerobic phenotype is shared with the PAO, in being able to assimilate substrates like acetate and use these to synthesize intracellular PHA. Then under aerobic conditions the GAO like the PAO are thought to metabolize this stored PHA, but now to synthesize intracellular glycogen (which unlike polyP and PHA is not routinely detectable by staining) instead of polyP as the PAO do. Not too surprisingly for organisms with such a phenotype, these GAO appear to be ubiquitous members of anaerobic: aerobic EBPR communities. They are viewed as potential competitors of the PAO for anaerobic substrate uptake and thus a likely cause of EBPR failure (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). As with the PAO, this working 'definition' of a GAO is somewhat unsatisfactory, especially since in practice the anaerobic component of the GAO phenotype is usually the only one determinable, and glycogen accumulation in activated sludge has never been demonstrated directly *in situ* for any putative identified GAO population. It is probable that EBPR populations exist which assimilate substrates like acetate and synthesize PHA anaerobically, but do not subsequently synthesize either glycogen or polyP aerobically. Equally it is likely that many populations with the GAO phenotype exist in EBPR processes, but whose identity is currently unknown (e.g., Oehmen *et al.*, 2006).

What do we know about the identity of the GAO?

For the same reasons that *Acinetobacter* was considered likely PAO, so were cultured alphaproteobacterial *Amaricoccus* once considered probable GAO (Seviour *et al.*, 2003). Yet they also lack the expected phenotype, showing no ability for anaerobic substrate assimilation or PHA production (Falvo *et al.*, 2001; Seviour *et al.*, 2003). Like *Acinetobacter* they too probably represent 'laboratory weeds', growing very well on artificial medium but largely irrelevant in nature. In the absence of being able to identify glycogen accumulating cells directly by staining (Serafim *et al.*, 2002), recognising the GAO in EBPR communities has been based on applying a range of molecular methods to identify the dominating populations (often as tetrad forming organisms or TFO) in systems with low EBPR capacity. Whether these possess the GAO phenotype can be determined by fluorescence *in situ* hybridization/microautoradiography (FISH/MAR) (Kong *et al.*, 2006; Ahn *et al.*, 2007; Wong and Liu, 2007).

It seems from these studies that the GAO are phylogenetically diverse bacteria, currently including the gamma-proteobacterial *Candidatus* 'Competibacter phosphatis' (Crocetti *et al.*, 2002), the alphaproteobacterial *Sphingomonas*-related organisms (Beer *et al.*, 2004) and *Defluviicoccus*- related organisms (Wong *et al.*, 2004; Maszenan *et al.*, 2005; Meyer *et al.*, 2006). FISH probes are available for each of these phylotypes. Allowing for how we define a GAO, *Dechloromonas* related *Betaproteobacteria* may also show the GAO phenotype in continuously aerated 'aerobic' lab-scale EBPR systems (Ahn *et al.*, 2007). Emphasizing how flexible we need to be in defining what a GAO (and PAO?) is comes from the work of Zhou *et al.* (2008). By supplying an enriched culture of *Accumulibacter* with feed containing acetate but no P anaerobically, they showed acetate was still assimilated into PHA, but without polyP degradation and P release, a distinctive feature of PAO. Instead, energy for PHA synthesis appeared to come from glycogen degradation, which is equally distinctive of the GAO. Thus, apart from yet again demonstrating how remarkably metabolically flexible *Accumulibacter* is (a feature probably necessary in EBPR activated sludge processes), by simply changing the feed conditions, a PAO phenotype switches to that of a GAO.

How diverse are the GAO?

The same questions raised earlier in relation to the *Accumulibacter* apply equally to the *Competibacter* and *Defluviicoccus* GAO, for the same reasons. 16S rRNA gene analysis has been popular in attempts to assess the diversity amongst these and the attempts to overcome the limitations of this approach are discussed here.

Undoubtedly even 16S rRNA sequence data reveal that the gammaproteobacterial GAO *Competibacter* are phyloge-

netically diverse (Kong *et al.*, 2002). Nielsen *et al.* (1999) first identified members of this group in a deteriorated EBPR system using DGGE analysis and designed two FISH probes, Gam1278 and Gam1019 to cover the dominant sequences found. Crocetti *et al.* (2002) who named this group '*Competibacter*' designed new probes, GAOQ989 and GAOQ431 from additional 16S rRNA sequence data they obtained, to cover all the then known sequences. However, the story did not finish there, and finding further sequence diversity encouraged Kong *et al.* (2002) to design a set of FISH probes now defining seven subgroups. An additional probe was designed to cover all the members of this so-called GB group, as not all were targeted by the GAOQ989 and GAOQ431 probes. Subsequent FISH/MAR studies using the probes they designed for each sub-group revealed only minor differences in their substrate assimilation profiles (Kong *et al.*, 2006), but did show that only members of sub-group 6 were capable of denitrification while sub-groups 1, 4, and 5 reduced nitrate only to nitrite (Kong *et al.*, 2006). Importantly, Kong *et al.* (2006) also reported that some *Competibacter* cells responding to the universal GB probe were not covered by any of the sub-group probes and the likelihood of the existence of further sub-groups and increased biodiversity. In addition, 16S rRNA sequences closely related to those of the GB group have also been recovered from EBPR plants that are not targeted by any of the existing sub-group probes (Wong and Liu, 2006).

Interestingly it was proposed that propionate as the carbon source for EBPR reduced competitive abilities of *Competibacter* GAO in lab-scale systems (Oehmen *et al.*, 2004). Yet MAR studies on samples from full-scale plants revealed they assimilated propionate with similar MAR signal intensity to the *Accumulibacter* PAO (Kong *et al.*, 2006). Although the propionate MAR signal reportedly varied between experiments when sub-group targeted probes were applied (Kong *et al.*, 2006) this was considered to relate to the physiological state of the cells rather than reflect any phenotypic differences. It is possible that individual populations within each sub-group might vary in their affinity for propionate. The lab scale studies of Oehman *et al.* (2004) did not apply the sub-group level FISH probes, leaving open the possibility of another sub-group of *Competibacter* different to those found in the full-scale EBPR studies of Kong *et al.* (2006). Nielsen *et al.* (1999) also indicated that some of these organisms assimilated phosphate, although this has not been reported since.

Whether the 16S rRNA gene can indicate the full biodiversity of this group is unclear. Some of the probes have been designed against a single sequence to define a subgroup, while the sequences making up other sub-groups vary by up to 4.7% in their 16S rRNA sequences. Can FISH be used to provide further delineation between subgroups or to target new sub-groups? There is no FISH probe available which targets GB sub-group 1, and relies for its *in situ* identification on a probe that covers sub-groups 1 and 2 whereby cells targeted by this probe and not by the probe specific for sub-group 2 are assumed to be from sub-group 1 (Kong *et al.*, 2002).

No obvious link was found between *in situ* physiology of the different sub-groups and their distribution in a survey involving 12 full-scale EBPR plants (Kong *et al.*, 2006). Understanding how individual sub-groups behave in response to different operational conditions is likely to be vital in attempts to understand and ultimately control the growth of this group of organisms.

Much less is known of the phylogenetic diversity among the *Defluviicoccus* GAO. This is due in part to difficulties in acquiring their 16S rRNA sequence information in clone libraries generated from EBPR communities even where they are abundant (Meyer *et al.*, 2006). Success has been achieved with prior enrichment (Wong *et al.*, 2004; Meyer *et al.*, 2006; Wong and Liu, 2007; McIlroy *et al*., submitted). Wong *et al.* (2004) first proposed that putative alphaproteobacterial GAO may be related to *Defluviicoccus vanus* from clone sequences in a library constructed with alpha-proteobacterial targeted PCR primers. These sequences formed a monophyletic group including *D. vanus.* The two FISH probes they designed from these (TFO_DF218 and TFO_DF618) targeted members of the entire cluster. Then Meyer *et al.* (2006) obtained further 16S rRNA sequences also related to *D. vanus,* which formed a distinct second cluster with 97% similarity between members, but sharing only 90% similarity to those of Wong *et al.* (2004). The probes Meyer *et al.* (2006) published (DEF988 and DEF1020) were also designed so that together they targeted the whole cluster. A third *Defluviicoccus* 'cluster' (Wong and Liu, 2007) is based on a single 16S rRNA sequence only from a non-EBPR system but no FISH probes are available to detect it. Additional sequence data increasing the diversity among members of both clusters I and II have been reported (Wong and Liu, 2007), but the FISH probe designed to cover these for cluster II members (TFO_DF629) does not appear to work (Wong and Liu, 2007; McIlroy *et al*., submitted). Furthermore, insufficient sequence data were obtained to determine whether the probes already available for the cluster II members (Meyer *et al.*, 2006), covered these additional sequences.

Application of the *Defluviicoccus* FISH probes in combination with MAR, to EBPR communities has indicated considerable morphological and physiological variation exists among these GAO (Wong *et al.*, 2004; Burow *et al.*, 2007; Wong and Liu, 2007; Schroeder *et al*., submitted). The aerobic substrate uptake profiles (Schroeder *et al.,* submitted) for cluster II members in an aerated novel EBPR process (see later) differed markedly to those reported by Burow *et al.* (2007) in the community of a full scale anaerobic: aerobic EBPR process. This was especially true with propionate, which was only very slowly assimilated in those identified by Burow *et al.* (2007), and is a surprising result, considering that their high apparent affinity for propionate was exploited to enrich them by RNA-SIP (stable isotope probing) (Meyer *et al.*, 2006). These studies suggest that the currently available FISH probes do not adequately indicate the full diversity within this group.

High similarity of 16S rRNA sequences in cluster II clone libraries recovered from communities of EBPR plants with very different operational conditions might question the suitability of 16S rRNA as a phylogenetic marker (McIlroy *et al.,* submitted). With the exception of the *D. vanus* sequence all the 16S rRNA data available for clusters I and

II *Defluviicoccus* were obtained from lab scale reactors fed either amino acids and/or simple fatty acids (McMahon *et al.*, 2002; Wong *et al.*, 2004; Zhang *et al.*, 2005; Meyer *et al.*, 2006; Wong and Liu, 2007; McIlroy *et al.*, submitted). Even with such a reactor containing almost all alphaproteobacterial TFO, Oehman *et al.* (2006) showed only 16% of its biomass hybridized with the existing *Defluviicoccus* probes or other probes designed to target known alphaproteobacterial TFO. Additional sequence information from a variety of EBPR-systems is likely to reveal a much higher level of diversity amongst this group of important organisms.

Which factors affect competition between the GAO and PAO?

Unfortunately many early publications describing how EBPR performance is altered in response to changes in plant operating conditions fail to describe any corresponding shifts in the microbial composition of the communities. This work is of little value if we seek to answer the question posed above, and so will not be discussed here. However, several parameters are now known which appear to affect relative PAO/GAO population sizes (Oehmen *et al.*, 2007), including for example, the carbon source. Its influence depends on which GAO population is involved, emphasizing further the problem with studies where GAO identity is not provided. Thus, while propionate favours *Accumulibacter* over the *Competibacter* GAO (Oehmen *et al.*, 2004), acetate is used by both (Oehmen *et al.*, 2007). On the other hand, indirect evidence suggests that the alpha-proteobacterial *Defluviicoccus* GAO assimilate propionate preferentially to acetate, unlike the *Accumulibacter* PAO which showed no clear preference for either (Oehmen *et al.*, 2007). By alternating both carbon sources in the feed, highly enriched cultures of *Accumulibacter* could be obtained (Lu *et al.*, 2006), and such a strategy was suggested as a tactic for discouraging the GAO and operating EBPR processes more reliably. Yet Dai *et al.* (2007) obtained a highly enriched community of cluster 1 (see below) *Defluviicoccus* GAO with only an acetate feed, and suggested that the low dissolved oxygen levels used (1% saturation) were selecting these GAO populations, again emphasizing how much is still to be learned about the conditions determining their proliferation.

Other published data suggest that EBPR systems operate best at pH>7.25 (Oehmen *et al.*, 2007), while at lower pH values the GAO have a competitive advantage over the PAO. However, little microbiological data exist to help us interpret such trends, although Oehmen *et al.* (2005) have shown that both *Competibacter* and unidentified (possibly novel) alphaproteobacterial TFOs became numerically less important as pH was increased from 7 to 8 in reactors fed acetate and propionate respectively.

Several reports have also suggested that EBPR capacity falls at higher temperatures (30°C) (Oehmen *et al.*, 2007), and indirect evidence has suggested that this also may result from changes in the PAO:GAO balance. Less well understood is how plant configuration and operating conditions might influence this relationship, or which GAO populations are favoured (Oehmen *et al.*, 2007).

What anaerobic metabolic differences exist between the GAO and PAO?

The metabolic properties of these phenotypes might be expected to influence their individual abilities to compete under anaerobic conditions for substrates. Bearing in mind that the PAO have both polyP and glycogen stores and the GAO only glycogen, how each generates reducing power and energy for PHA production is likely to differ. How they then balance their intracellular redox under anaerobic conditions has also been controversial (Oehmen *et al.*, 2007). The whole genome sequences of *Accumulibacter* (García Martín *et al.*, 2006) reveal some (but maybe not all) answers to these questions, since many functionally important genes await characterization, and incidentally contain several real surprises. In the past such questions have attracted considerable interest and generated much controversy and debate. Targeted metabolic inhibitor studies, with highly enriched but not pure cultures of *Accumulibacter* and *Defluviicoccus* (cluster 1) exploiting this genomic information (Saunders *et al.*, 2007; Burow *et al.*, 2008) have also allowed speculation on their respective competitive abilities under EBPR conditions. Similar data are not yet available for *Competibacter* or cluster 2 *Defluviicoccus*. A few examples of these metabolic features will be discussed briefly here.

It now seems clear from genome sequence data that under anaerobic conditions *Accumulibacter* has what is required to operate a functional anaerobic TCA cycle, including a unique (?) cytochrome b/b_6 allowing succinate dehydrogenase to function anaerobically (García Martín *et al.*, 2006). It also degrades its glycogen stores not with the Entner Doudoroff (ED) pathway, as once considered likely (Oehmen *et al.*, 2007), but by the Embden Meyeroff Parnas (EMP) pathway (García Martín *et al.*, 2006), an important difference, since ATP yields from the former are lower than those from the latter. On the other hand, ^{13}C-NMR data from an acetate fed enriched culture of both *Defluviicoccus* and *Competibacter* (Lemos *et al.*, 2007) suggest both might employ the ED pathway for their anaerobic glycogen degradation.

Both Saunders *et al.* (2007) and Burow *et al.* (2008) produce persuasive evidence from their metabolic inhibitor studies with highly enriched cultures that *Accumulibacter* assimilates acetate by a process of active transport involving an acetate permease, although some facilitated diffusion may also be involved. The necessary proton motive force (pmf) or membrane potential is generated not by any ATP dependent mechanism, but by P efflux via a Pi transporter, and part of the pmf seems to be conserved by the cells as ATP by influx via a H^+-ATPase. Since its inhibition had no effect on acetate assimilation, Burow *et al.* (2008) suggested fumarate reductase had no role in generating this pmf, and any carbon flux through the reductive branch of the TCA cycle (Fig. 1) (Oehmen *et al.*, 2007) was negligible. Nor did a sodium membrane potential (smf) play any role in anaerobic acetate transport.

The anaerobic metabolic attributes of cluster 1 *Defluviicoccus* appear to differ markedly to those of *Accumulibacter* in these key steps in EBPR (Burow *et al.*, 2008). Thus, acetate uptake rates appear to be slower in *Defluviicoccus* and both a pmf and smf are involved in its transport. Furthermore, since inhibition of fumarate reductase markedly in-

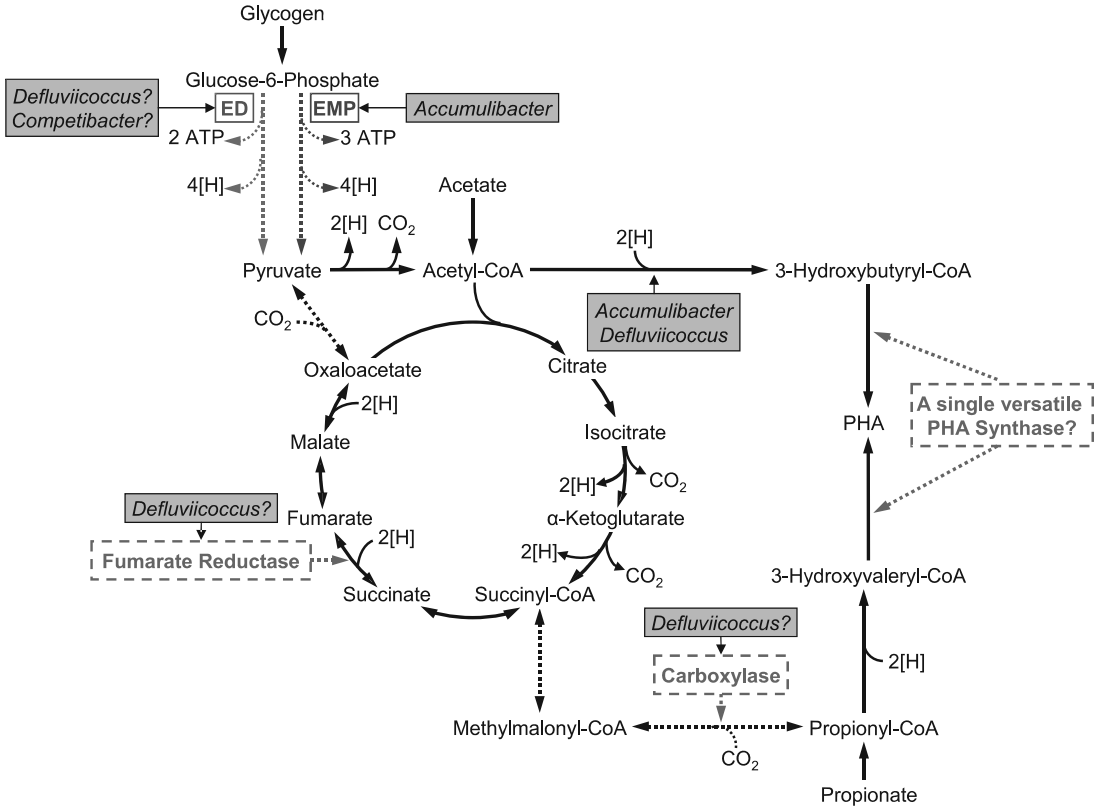

Fig. 1. Anaerobic metabolic differences between the *Accumulibacter* PAO, and *Defluviicoccus* GAO based on the inhibition studies of Burow *et al.* (2008). Adapted from Oehmen *et al.* (2007).

hibited acetate transport in *Defluviicoccus*, considerable carbon efflux probably occurs through the reductive branch of the TCA cycle, involving fumarate reductase and the succinate-propionate pathway (also see Lemos *et al.*, 2007). Also in the *Defluviicoccus* enriched community a much higher hydroxyvalerate (HV):hydroxybutyrate (HB) ratio was measured than in that dominated by *Accumulibacter,* (where incidentally only a single versatile phaC synthase gene is seen; García Martín *et al.*, 2006), consistent with this. Thus, in *Defluviicoccus*, the pmf is generated quite differently, instead involving H^+ efflux and fumarate reductase, or Na^+ efflux, and some energy conserved by cells from its influx through ATPase (Burow *et al.*, 2008).

Based on these results, Burow *et al.* (2008) have suggested that the scavenging ability of the *Accumulibacter* acetate permease should provide these populations with a selective advantage over *Defluviicoccus* in full scale plants, where acetate levels are likely to be generally low. Thus, the view that EBPR processes fail because the PAO are out-competed for acetate anaerobically by the GAO may need closer examination, and the influence of parameters like pH and temperature on EBPR viewed more widely. Whether this statement applies to *Competibacter* GAO is so far unknown. Burow *et al.* (2008) also suggest that maintaining low acetate levels by changing the feed strategy from a dump feed to a trickle feed would disadvantage *Defluviicoccus,* and might allow EBPR plants to function more stably. Clearly further metabolic information, especially which factors might regulate key enzyme synthesis and activity, is required before we can predict such events confidently, as is the need to understand more completely the possible levels of metabolic diversity existing between strains of these populations (e.g., cluster 1 and cluster 2 *Defluviicoccus*?).

What aerobic metabolic differences exist between the GAO and PAO?

Genome sequence data (García Martín *et al.*, 2006) also reveal that *Accumulibacter* populations possess genes encoding two transport systems functioning in aerobic P uptake, a low affinity (Pit) and high affinity (Pst) system allowing scavenging of Pi at very low concentrations. Under the aerobic conditions used by Burow *et al.* (2008), Pst seemed to function continuously at both high and low P levels, and importantly at high velocity. In the absence of similar whole genome sequence data it is difficult to predict how *Defluviicoccus* might regulate the metabolism of its stored PHA aerobically, again via the ED pathway and synthesizing glycogen at the same time probably from pyruvate and glyceraldehyde 3-phosphate (Lemos *et al.*, 2007), or whether it, like *Accumulibacter,* might hold a few metabolic surprises.

How crucial is the anaerobic feed phase in EBPR?

As stated earlier, the view is generally held that EBPR processes will only operate if the biomass is cycled continu-

ously through anaerobic:aerobic zones for the reasons explained earlier. In other words all agree that an anaerobic feed stage is essential (Seviour *et al.*, 2003; Oehmen *et al.*, 2007). Why this is so is not clear. The reason generally given is that if oxygen or nitrate/nitrite is present, then other aerobic or denitrifying heterotrophic organisms may (like the GAO) out-compete the PAO for substrates like acetate because they can use them for respiration and growth. Yet where is the evidence for this? We know of no published microbiological data to support such arguments. Furthermore, the metabolic data discussed above would suggest that *Accumulibacter* possess a very effective system for anaerobic acetate assimilation, which of course is not to say that other organisms may not equal or surpass it. In fact Ahn *et al.* (2007) showed that when the supply of C (acetate) and P to a sequencing batch reactor (SBR) were temporally separated, then reliable and complete EBPR was achieved. Aerobic P release paralleling acetate uptake occurred, which in the absence of any exogenous P supply was used largely to synthesize intracellular PHA and not cell growth. Once all the acetate had been assimilated, cells then respired their stored PHA and the P released earlier was completely taken up to support cell growth. In the absence now of any exogenous P, once P was added in the subsequent famine stage, it rapidly disappeared from the medium, coinciding with further PHA degradation. The major PAO involved in this process were identified as *Accumulibacter*, and cluster 2 *Defluviicoccus* were also present.

It might be argued that the measured fall in dissolved oxygen levels in the medium (to about 10~20% saturation and to a redox of 100~50 mv) coinciding with acetate uptake was sufficient to create anaerobic microenvironments within the flocs, explaining the 'aerobic' P release. Microelectrode studies are needed to confirm or otherwise this suggestion, although several other reports suggest that aerobic P release exactly as described by Ahn *et al.* (2007), does occur. Thus, Guisasola *et al.* (2004) described precisely the same sequence of events in their study when biomass from an anaerobic: aerobic EBPR process was run aerobically, although no microbiological data were provided to suggest which the PAO were. Pijuan *et al.* (2005) showed essentially the same aerobic behaviour in a community dominated by *Accumulibacter*. They also showed (Pijuan *et al.*, 2006) that when an anaerobic:aerobic EBPR process (where carbon and P are added simultaneously) was converted to a fully aerobic process (both feed and famine stages were operated aerobically), EBPR capacity was retained for several days before failing. FISH analyses showed no major changes in the number of *Accumulibacter* and *Competibacter* upon failure, suggesting that it was not associated with any major changes in the key populations in the community composition. Instead it appeared to be a consequence of the PAO adjusting their metabolism to aerobic conditions. Thus, when Pijuan *et al.* (2006) measured the chemical transformations taking place in this now 'aerobic' process, major shifts in the activities of the community had occurred. Although PHA production did not change following EBPR failure, a critical difference was the gradual decrease in the turnover rate of the stored glycogen during the feed and famine stages, and in the $P_{uptake}/P_{release}$ ratio, where less and less P was taken up with repeated aerobic FEED periods. They concluded that not only could *Accumulibacter* survive under aerobic conditions, but it was in fact very competitive in terms of its ability to assimilate acetate, raising doubts about the earlier predictions. The data suggested the PAO were metabolizing acetate aerobically to provide energy via the TCA cycle to synthesize PHA without having to use any of their stored glycogen as happens in anaerobic: aerobic EBPR processes. Similar features were demonstrated by the community in the aerobic process of Ahn *et al.* (2007), suggesting that the PAO and GAO populations were coping with those conditions, and again showing just how remarkably metabolically versatile *Accumulibacter* is.

Growing these PAO and GAO in pure culture

This work of Burow *et al.* (2008) described above is clearly valuable in any attempt to understand the *in situ* physiology of these EBPR populations, but there are always doubts about the absolute specificities of the inhibitors used in such studies. Furthermore although unlikely, there is the additional risk with enrichment cultures like those they used for *Accumulibacter*, and *Defluviicoccus*, that other populations present or physiological diversity between strains of FISH defined phylotypes may affect the metabolic outcomes. In our view, the advantages of having pure cultures of both PAO and GAO available for work of this kind are incalculable. Interpreting their whole genome sequence data would be less equivocal, factors affecting regulation of synthesis and activity of key enzymes could be more readily elucidated, gene knockout studies could be carried out and their genetic systems resolved. The EBPR literature is full of phrases suggesting that many attempts have been made to grow these, yet is equally deficient in details of the methods used and their outcomes. Only the GAO *Defluviicoccus* has been grown in the laboratory (Maszenan *et al.*, 2005), and this strain was phylogenetically quite distinct from those detected in activated sludge by culture independent methods.

Certainly the genome sequence data from *Accumulibacter* provide clues as to how this might be planned. Thus, very unexpectedly, *Accumulibacter* possesses all the genes needed for nitrogen and carbon dioxide fixation, although whether these would be expressed in carbon and nitrogen rich activated sludge systems is unlikely. A high requirement for cobalt is also indicated. However, García Martín *et al.* (2006) tease the reader by saying that this information generated enriched but not pure cultures. What was not relayed was how enriched the developed community was? Which other populations also grew under the imposed conditions? Were they closely related populations? Under what conditions was this enrichment carried out (presumably inoculating cells from such an enriched culture onto N free medium in a CO_2 enriched environment with acetate and/or propionate as carbon source)? Did the eventual community fix nitrogen? Achieving this task of culturing these populations is so important that others should persist, as the scientific rewards from success would be substantial. Until whole genome sequence data are available for *Defluviicoccus*, no similar rational approach to its culture can be undertaken.

How widely distributed in nature are these *Accumulibacter*? As already mentioned, the possession of genes encoding all the enzymes etc required for fixation of nitrogen and CO_2 and hence indicating no requirement for organic forms of either nitrogen or carbon for their growth, shows these are organisms likely to be competitive and to thrive in nutrient deficient habitats. Thus, as García Martín *et al.* (2006) state, they seem to be well suited to freshwater environments and sediments in lakes and streams. Having reliable, sensitive and specific PCR based methods for their detection makes this a fairly straightforward task. One might expect it to reveal how phylogenetically diverse *Accumulibacter* populations really are, as well as assist in explaining under which conditions each phylotype might be favoured. Remember, the genome sequence data came from populations growing in lab-scale reactors fed synthetic medium. It seems likely that those obtained from more complex habitats would differ markedly from these.

Conclusions

This brief article has tried to outline what we currently know about EBPR and the key microbial populations found there, and how having the genome sequence of *Accumulibacter* has helped. We can look into the crystal ball, and based on the remarkable advances in EPBR microbiology which have occurred in the last decade, make a few predictions of what might happen in the next decade or so, accepting that we are almost certain to be widely inaccurate in our predictions. Nevertheless, by then we may have;

i) Understood why the PAO store polyP. Is it necessary to enable them to survive anaerobic conditions in the absence of any other means to generate energy for substrate assimilation, as Burow *et al.* (2008) suggest? Or does it have some other function, or is it a consequence of breakdown in metabolic regulation?

ii) Isolated into pure culture *Accumulibacter*, *Defluviicoccus*, and *Competibacter* strains from different habitats using novel isolation methods, and gained a better understanding of their ecology and phylogeny, genetics and physiology/biochemistry

iii) Applied this information together with functional microarrays to allow us to predict their behaviour in EBPR systems, and strategies to manipulate them

iv) As part of this, developed improved EBPR plant configurations, especially in how we operate the feed: famine stages

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